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Neurogenesis in the spider *Cupiennius salei*

Angelika Stollewerk*, Mathias Weller and Diethard Tautz

Abteilung fuer Evolutionsgenetik, Institut fuer Genetik, Universitaet zu Koeln, Weyertal 121, 50931 Koeln, Germany
*Author for coprrespondence (e-mail: angelika.stollewerk@uni-koeln.de)

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SUMMARY

To uncover similarities and differences in neurogenesis in arthropod groups, we have studied the ventral neuroectoderm of the spider *Cupiennius salei* (Chelicerata, Aranea, Ctenidae). We found that invaginating cell groups arose sequentially, at stereotyped positions in each hemisegment and in separate waves, comparable with the generation of neuroblasts in *Drosophila*. However, we found no evidence for proliferating stem cells that would be comparable with the neuroblasts. Instead, the whole group of invaginating cells was directly recruited to the nervous system. The invagination process is comparable with *Drosophila*, with the cells attaining a bottle-shaped form with the nuclei moving inwards, while actin-rich cell processes remain initially connected to the surface of the epithelium. This general pattern is also found in another spider, *Pholcus phalangioides*, and appears thus to be conserved at least among the Araneae. We have identified two basic helix-loop-helix encoding genes – *CsASH1* and *CsASH2* – that share sequence similarities with proneural genes from other species. Functional analysis of the genes by double-stranded RNA interference revealed that *CsASH1* was required for the formation of the invagination sites and the process of invagination itself, whereas *CsASH2* seemed to be required for the differentiation of the cells into neurones. Our results suggest that the basic processes of neurogenesis, as well as proneural gene function is conserved among arthropods, apart of the lack of neuroblast-like stem cells in spiders.

Key words: Neurogenesis, Proneural genes, Invagination, Mitosis, Chelicerate, *Cupiennius salei*

INTRODUCTION

The phylum arthropoda comprises four classes: myriapoda, crustacea, insecta and chelicerata. The monophyly or polyphyly of the arthropoda has been questioned, as has the relationship of the individual classes to each other. The principle difficulty involves deciding which of the morphological similarities that unite these groups are the result of close evolutionary relationships and which represent the convergence of traits. Today, most authors treat the Arthropoda as a monophyletic group based on morphological (Weygoldt, 1985; Lauterbach, 1973) and molecular data (Friedrich and Tautz, 1995; Tuberville et al., 1991; Ballard et al., 1992). Furthermore, it has been shown that insects are not closely related to myriapods, as was originally assumed. Rather, the insects seem closely related to crustaceans (Friedrich and Tautz, 1995; Boore et al., 1995). The chelicerates represent a basal arthropod group and are probably a sister group of the myriapods (Friedrich and Tautz, 1995; V. W. Hwang, M. Friedrich, D. T., C. J. Park and W. Kim, unpublished).

The comparative analysis of developmental processes is a powerful method to unravel phylogenetic relationships. By comparing the expression patterns of genes involved in segmentation and segment identity in holometabolous (Sander, 1988) and hemimetabolous (Akam et al., 1988; Patel et al., 1989a; Patel et al., 1989b) insects as well as crustaceans (Abhzanov and Kaufman, 1999; Abhzanov and Kaufman, 2000) and chelicerates (Damen et al., 2000; Abzhanov et al., 1999; Damen and Tautz, 1998; Damen et al., 1998; Telford and Thomas, 1998), new insights into the evolutionary relationships of the different arthropod taxa have been gained. In addition, the morphological comparison of neurogenesis in insects and crustaceans has supported the molecular evidence of a sister group relationship between these two classes: both in insects and crustaceans basally located ganglion mother cells arise by unequal divisions of neural stem cells (Pasakony, 1994; Scholz, 1990; Scholz, 1992; Dohle and Scholz, 1988; Harzsch and Dawirs, 1996). In myriapods, however, which were formerly assumed to be closely related but more basal to the insects, no neuroblasts can be detected (Whitington et al., 1991; Weygoldt, 1985; Anderson, 1973).

Within the arthropods, neurogenesis is best analysed in locusts (Bate, 1976; Bate and Grunewald, 1981) and *Drosophila melanogaster* (Hartenstein and Campos-Ortega, 1984). The ventral neuroectoderm consists of a sheet of ectodermal cells, of which about 25 cells per hemisegment delaminate into the embryo as neural precursor cells, called neuroblasts. Delamination takes place in five discrete pulses. The remaining cells of the neurogenic region generate the ventral epidermis. The neuroblasts form in a highly stereotyped temporal and spatial pattern (Hartenstein et al., 1987; Doe et al., 1988; Jiménez and Campos-Ortega, 1990; Campos-Ortega and Haenlin, 1992; Goodman and Doe, 1993). Based on the analysis of mutants, several *Drosophila* genes

have been identified that regulate the decision between epidermal and neural fate (Campos-Ortega, 1993; Ghysen et al., 1993; Muskavich, 1994). The competence to take on neural fate depends on the proneural genes (Cabrera et al., 1987; Romani et al., 1989; Jiménez and Campos-Ortega, 1990; Cubas et al., 1991; Martin-Bermudo et al., 1991; Skeath and Carroll, 1992). In embryos homozygous for loss-of-function mutations in the proneural genes, neuroblasts are missing (Jiménez and Campos-Ortega, 1990), whereas ectopic expression of proneural genes gives rise to additional neural precursor cells (Campuzano et al., 1986; Brand et al., 1993; Hinz et al., 1994). The proneural genes *achaete*, *scute* and *lethal of scute*, together with *asense*, form the so-called *achaete-scute* complex (ASC). These genes code for related proteins that contain a basic helix-loop-helix (bHLH) domain characteristic for a family of transcriptional regulators (Murre et al., 1989; Murre et al., 1994). In groups of cells that express these proneural genes – the proneural clusters – proneural gene expression becomes restricted to one cell of the cluster, the future neuroblast, by the activity of the neurogenic genes. This process is called lateral inhibition and is mediated by the neurogenic genes *Notch* and *Delta*. Mutations that affect the process of lateral inhibition lead to an overproduction of neurones – a neurogenic phenotype (Lehmann et al., 1981; Lehmann et al., 1983).

Much less is known about neurogenesis in the remaining arthropods. As has been mentioned above, neurone precursor cells that have many of the characteristics of insect neuroblasts could be detected in crustaceans, although it is not clear whether they are homologous to the insect neuroblasts. In contrast to insects, crustacean neuroblasts do not delaminate from the surface layer of neuroectodermal cells before or during their divisions, and they are not associated with the specialized sheath cells found in insects (Doe and Goodman, 1985a; Doe and Goodman, 1985b; Doe and Goodman, 1985c; Scholtz, 1992). In addition, at least some crustacean neuroblasts can give rise to epidermal cells after they begin to bud off ganglion mother cells (Dohle and Scholz, 1988).

The comparison of neurogenesis in myriapods and insects has led to the assumption that this developmental process is less conserved in arthropods. The formation of the ventral ganglia in myriapods is associated with the formation of ‘ventral organs’; these are shallow pits that develop within the ectoderm external to the ganglia and, in some cases, are subsequently incorporated as cavities into the ganglion (Whitington and Bacon, 1997). Furthermore, the earliest axon pathways of myriapod embryos arise by the posteriorly directed growth of axons that originate from neurones located in the brain, rather than from segmental neurones, as in insects (Whitington et al., 1991).

Apart from a few classical accounts, neural development in the chelicerates has received little attention. In xiphosurans, and most scorpions and arachnids, neurogenesis occurs by a generalized inward proliferation of neuroectodermal cells to produce paired segmental thickenings (Anderson, 1973). Neuroblasts have been described for three chelicerate species, but it is possible that the data were partly misinterpreted, owing to technical limitations at the time (Winter, 1980; Mathew, 1956; Yoshikura, 1955). The neurogenesis of *Cupiennius salei* (Chelicerata, Arachnida, Aranea, Ctenidae) has not been analysed until now.

MATERIALS AND METHODS

Cupiennius salei stock

Fertilized females of the Central American wandering spider *Cupiennius salei* Keyserling (Chelicerata, Arachnida, Araneae, Ctenidae) were obtained from a colony bred by Ernst-August Seyfarth in Frankfurt am Main, Germany and from our newly established colony bred in Cologne. Embryos were collected as described before (Damen et al., 1998).

PCR cloning

CsASH1 and *CsASH2* were initially found by RT-PCR on RNA prepared from germband embryos, using degenerate primers directed against conserved positions in the basic region and second helix of the bHLH domain of five invertebrate and three vertebrate proneural genes. We used the following primers: ASCUL1, MGNAA-YGMNMGNGARMGNAA; ASCUL2, GMNMGNGARMGNAA-NMGNGT; ASCUL3, MGNGTNRANYWNGTNAA; ASCULre1, ACYTTNSWNADYTTYTT; ASCULre2, ARNGTNTCYACYTT-NSWNADYTTYTT. ASCUL2 and ASCULre2 were used for a nested PCR on an 1 μl aliquot of the initial PCR. The obtained PCR fragments were cloned and sequenced. Larger fragments for both genes covering the complete ORF were obtained by rapid amplification of cDNA ends (Marathon cDNA amplification kit, Clontech). The sequences obtained were deposited in the EMBL/GenBank/DDBJ databases (Accession Numbers, AJ309490 and AJ309491).

In situ hybridization

Whole-mount in situ hybridizations were performed as described (Damen and Tautz, 1999a; Damen and Tautz, 1999b).

Phalloidin staining of embryos

Phalloidin staining of spider embryos was performed as has been described for flies (Stollewerk, 2000).

YOYO staining of embryos

YOYO-1 was purchased by Molecular Probes, the Netherlands. Before staining the embryos were incubated for 2 hours in 10 μl RNaseA (Roche, Mannheim, Germany). After several washes in phosphate-buffered saline (PBS), the embryos were incubated in 1 μl YOYO per ml PBS for 1 hour.

Antibody staining

Immunohistochemistry was performed as described previously (Klämbt et al., 1991; Mitchison and Sedat, 1983). Anti-Phospho-Histone 3 (PH3) antibody was provided by F. Sprenger (Institut for Genetics, Cologne). Anti-Horseradish peroxidase antibody was purchased from Dianova, Hamburg.

Double-stranded RNA interference

Preparation of double-stranded RNA, injection and further treatment of the embryos were performed as described previously (Schoppmeier and Damen, 2001; DOI10.1007/s004270000121) with one modification: double-stranded RNA corresponding to the nucleotide positions 299 to 911 of the ORF of the pEGFP gene (provided by M. Gajewski; Institute for Genetics, Cologne) was used for control injections.

RESULTS

The embryonic segments of *Cupiennius salei* arise sequentially. At first, the germband develops aggregations of cells that form the cephalic lobe and the caudal lobe. The cephalic lobe then divides to give rise to a cheliceral segment,

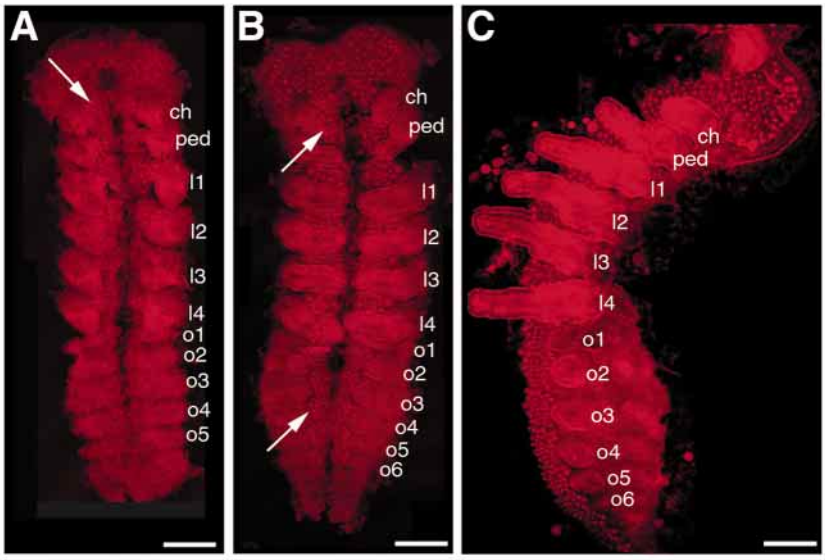

Fig. 1. (A-C) Sequential formation of invagination sites in the ventral neuroectoderm of *Cupiennius salei* embryos stained with phalloidin-rhodamine. (A) Confocal micrograph of a flat preparation of an embryo 130 hours after egg laying. Limb buds have developed in the prosoma (ch, ped, l1 to l4). The first five to eight invagination sites (arrow) are visible in the prosomal hemisegments and the first opisthosomal hemisegment. No invagination can be seen in the remaining opisthosomal hemisegments. (B) Three additional segments have been generated by the posterior growth zone 175 hours after egg laying. Abdominal buds are visible on the second to fifth opisthosomal hemisegments (o2 to o5). New invagination sites are not only added posteriorly but also anteriorly (arrows). (C) Left half of the germ band of a 200 hour embryo. At this stage 9 opisthosomal segments are visible. 30 to 32 invagination sites can be detected in each hemisegment of the prosoma and the opisthosoma. *Ch*, cheliceral segment; *ped*, pedipalpal segment; *l1* to *l4* walking legs 1 to 4, corresponding to prosomal segments 3 to 6; *o1* to *o6* opisthosomal segments 1 to 6. Scale bars: 200 μm.

whereas the remaining segments are formed by the posterior growth zone. When the germband has expanded to the point that the caudal and cephalic lobe almost touch each other, a furrow forms that divides the embryo into left and right parts that are connected only at the anterior and posterior points. The two sides move laterally until they finally meet in the dorsal midline (Seitz, 1966). This process is called inversion or reversion. Until now it was not known at what time during embryogensis that neurogenesis occurred in *Cupiennius salei.* In addition, the exact borders of the ventral neuroectoderm have not been described. To facilitate the morphological analyses of the neuroectodermal cells, embryos that were clearly segmented and already had limb buds in the prosoma (cephalothorax) and the opisthosoma (abdomen) were analysed first (approx. 175 hours after egg laying; Seitz, 1966).

Formation of invagination sites

Cupiennius embryos were stained with phalloidin-rhodamine, a dye that stains the actin cytoskeleton. Because the actin filaments stained by this agent are found mainly in the cortex of the cells, this technique can be used to investigate cell shapes in the confocal laser-scanning microscope (LSM). We made flat preparations of the stained embryos and scanned them from apical to basal using the LSM. At this time point, the ventral neuroectoderm of the spider consists of one single cell layer. In all embryos analysed, dots of high phalloidin staining could be detected in apical optical sections of the cephalic lobe, in all segments of the prosoma and in the first six opisthosomal segments between the edge of the limb buds and the ventral furrow (Figs 1B, 2A). Analysis of the ventral neuroectoderm at a higher magnification revealed that groups of five to nine basally enlarged cells were located underneath the strongly stained dots (Fig. 2B, arrows). Transverse sections show that these cells have a bottle-like shape (Fig. 2C,D). The cell nuclei are located basally (Fig. 2D, asterisks), whereas the bundled

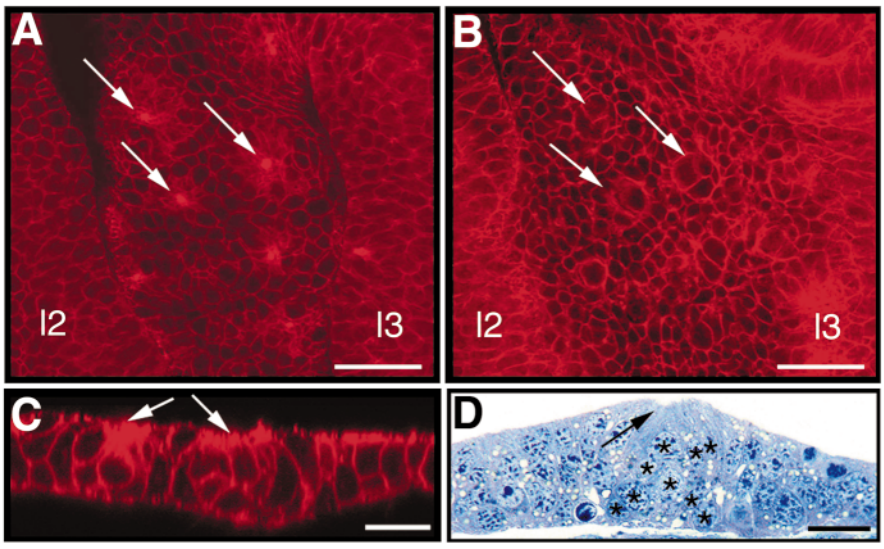

Fig. 2. (A-D) Morphology of the invagination sites. (A) Confocal micrograph of an apical optical section of the ventral neuroectoderm between leg 2 and leg 3 of an 175 hour embryo stained with phalloidin-rhodamine. The arrows point to dots of high phalloidin staining. (B) Basal optical section of the same region of the ventral neuroectoderm shown in A. The arrows point to groups of basally enlarged cells that are located underneath the dots of high phalloidin staining. (C) Confocal micrograph of a transverse optical section through two invagination sites (arrows). The cell processes of the basally enlarged cells extend to the apical surface. (D) Light micrograph of a transverse section through an invagination site. This invagination site consists of approximately nine cells (asterisks). The cell processes extend to the apical surface (arrow). Scale bars: 40 μm in A,B; 20 μm in C,D.

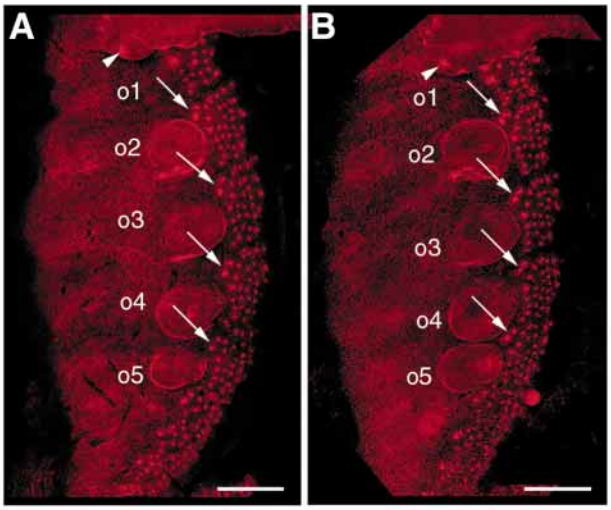

Fig. 3. (A,B) Stereotyped pattern of invagination sites. (A,B) Confocal micrographs of the opisthosoma of two different embryos stained with phalloidin-rhodamine. The invagination sites occupy the same positions in each hemisegment. The arrows point to two invagination sites that are located at the lateral anterior edge of each hemisegment. The arrowheads point to high phalloidin staining in the PNS. *o1* to *o5* opisthosomal segments 1 to 5. Scale bars: 200 μm.

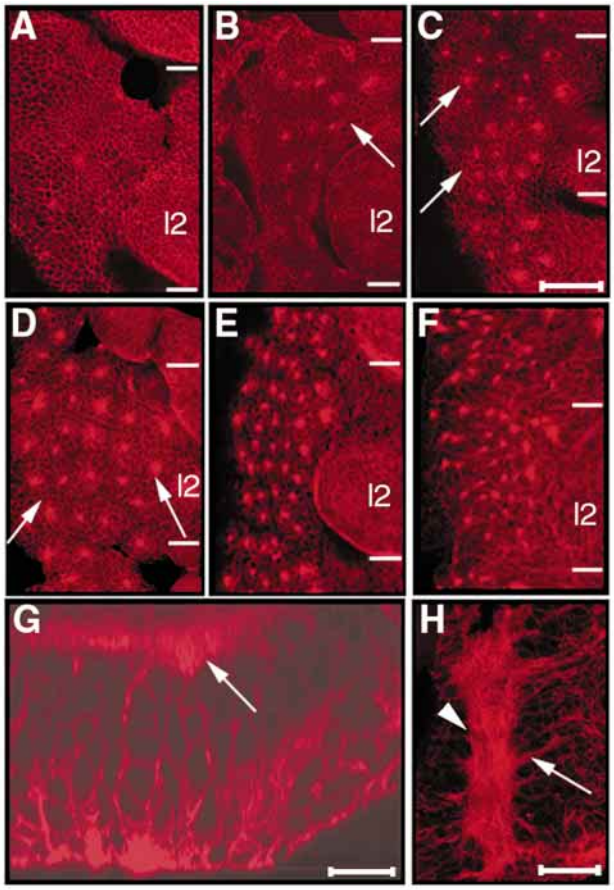

Fig. 4. (A-H) Confocal micrographs of the prosomal hemisegment corresponding to leg 2 (fourth prosomal segment) of embryos stained with phalloidin-rhodamine. Anterior is towards the top, the medial furrow towards the left. The white lines indicate the segmental borders. (A) No invagination sites can be detected up to 120 hours after egg laying. The limb buds are already visible. (B) At 130 hours, the first five to eight invagination sites arise in the anterior most lateral region of the prosomal and the first opisthosomal hemisegments (arrow). (C) At 150 hours, nine to 12 new invagination sites have formed posteriorly and medially to the anterior region, where the first invagination sites occurred (arrows). (D) At 175 hours, the next five to eight invagination sites are visible laterally and medially in the posterior region of the hemisegment (arrows). (E) At 190 hours, seven to ten invagination sites have been added between row three and four and at the posterior end of the hemisegment. (F) At 220 hours, the number of invagination sites decreases. (G) Confocal micrograph of a transverse section through the neuroectoderm of the fourth prosomal segment at 240 hours. Apical is at the bottom. At this time a growing neuropil can be detected basally (arrow). (H) Horizontal optical section through the neuropil of the fourth prosomal segment at 240 hours. The arrowhead points to the neuropil. The outgrowing axons of the invaginated neuroectodermal cells join the developing neuropil (arrow). In C-F, the legs were removed or put to the side. *l2*, walking leg 2 (corresponding to the fourth prosomal segment). Scale bars : 50 μm in A-F; 20 μm in G; 50 μm in H.

cell processes extend to the apical surface (Fig. 2D, arrow). These observations suggest that the apical dots of high phalloidin staining result largely from the constricted apical surfaces of these cell groups. Based on the morphology and the position of the cell groups, it can be concluded that these cells are invaginating neuroectodermal cells. Analysis of the precise arrangement of the sites of invagination in slightly older embryos revealed that they occupied the same anteroposterior and mediolateral positions within each hemisegment of all embryos analysed (Fig. 3, arrows). At that stage, there are seven rows of invagination sites, which consist of four to five invagination sites each.

To analyse the formation of the invagination sites in detail, embryos of different stages (130 hours to 220 hours (Seitz, 1966)) were stained with phalloidin-rhodamine and prepared for examination in the LSM. At 100-120 hours, the limb buds start to form in the prosoma and four opisthosomal segments have been formed by the posterior growth zone. At that time point there are no invagination sites visible in the neuroectoderm (Fig. 4A). The first invagination sites do not arise until 130 hours, within the cephalic lobe and the ventral neuroectoderm. At that time, five to eight invaginations sites form per prosomal hemisegment (Fig. 4B) and in the first opisthosomal hemisegments, whereas in the remaining opisthosomal segments, no invaginating cells can be detected (Fig. 1A). About 20 hours later, the abdominal buds are visible and two additional opisthosomal segments have been released from the posterior growth zone. At that time 16-17 invagination sites can be detected in the prosomal hemisegments (Fig. 4C). In the first to fifth opisthosomal hemisegments five to eight invagination sites are visible; in the sixth, only two. No invaginating cells can be detected in the last opisthosomal hemisegments. At 180 hours, two additional segments have been added to the opisthosoma. At that time about 25 invagination sites can be counted in all prosomal hemisegments (Fig. 4D). In the first to fifth opisthosomal hemisegments, there are 16 to 17 invagination sites; in the sixth, five to eight; and in the seventh, only two. The remaining opisthosomal hemisegments do not have any

invaginating cell groups. About 10 hours later, the number of invagination sites has increased again in every hemisegment. In the prosomal hemisegments 30 to 32 invagination sites can be detected (Fig. 4E); in the first to fifth opisthosomal hemisegment, about 25; in the sixth and seventh opisthosomal hemisegment, 16 to 17; and in the eighth, five to eight. The maximum of 30 to 32 invagination sites is reached by all hemisegments after about 200 hours after egg laying. Only a few hours later, the number of invagination sites decreases (Fig. 4F). The neuroectoderm has developed a second cell layer which consists of cells that have already invaginated (Fig. 4G) After about 220 hours, a growing neuropil can be detected basally (Fig. 4G,H, arrow, arrow head). These data show that there is an anterior-to-posterior gradient for the formation of invagination sites. Although in the prosoma, the same number of invagination sites can always be detected in all segments, the invagination sites in the opisthosoma are formed later in more posterior segments. This might be due to the fact that new segments are generated during the time of neurogenesis. In addition, the results reveal that invagination sites are not only added posteriorly, rather, additional invaginating cell groups arise in anterior segments four times during neurogenesis. It can therefore be concluded that there are four waves of formation of invagination sites in *Cupiennius salei*, which are comparable with the waves of neuroblast delamination in *Drosophila*.

Invaginating cells express HRP antigen

In an attempt to test whether the invaginating cells are clearly neural cells, we stained embryos that had developed all invagination sites with the anti-horseradish peroxidase antibody. It has been shown that this antibody binds to neurones in all other arthropods (insects, Jan and Jan, 1982; crustaceans, Meier and Reichert, 1990; myriapods, Whithington et al., 1991). At the stage analysed, the antibody stains 30 to 32 invagination sites per hemisegment in the ventral neuroectoderm of *Cupiennius* embryos, as well as the invagination sites of the cephalic lobe in the same pattern as phalloidin (Fig. 5, arrows). In addition, outgrowing axons are stained by the anti-HRP antibody (Fig. 5B, arrowhead). Analyses of younger embryos revealed that the HRP antigen is already expressed in the invaginating cells of embryos that have formed the first five to eight invagination sites per hemisegment. These data confirm the assumption that the invaginating cells are neuronal cells.

Invagination sites in *Pholcus phalangioides*

As the pattern of invagination sites in the ventral neuroectoderm of *Cupiennius salei* has not been described up to now it was interesting – and, from an evolutionary point of view, essential – to examine whether this pattern is reasonably conserved, at least in the order of spiders (Araneae). The Araneae are usually divided into three suborders: the Mesothelae, the Mygalomorphae and the Araneomorphae (Foelix, 1996). *Cupiennius* is a genus of Central American hunting spiders that belong to the suborder Mygalomorphae. Given that more than 90% of all spiders belong to the Araneomorphae (Foelix, 1996), we decided to analyse the neuroectoderm of the web spider *Pholcus phalangioides*, which is grouped into this suborder and can be found in dry, warm cellars in Central Europe. We collected cocoon bearing

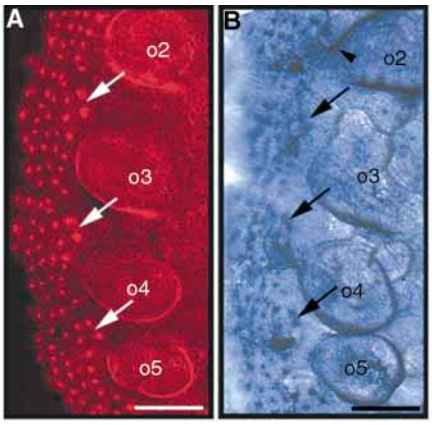

Fig. 5. (A,B) Invaginating cells express HRP antigen. (A) Confocal micrograph of the opisthosoma of a 200 hour embryo stained with phalloidin-rhodamine. All 30 to 32 invagination sites are visible in the opisthosomal hemisegments. The arrows point to the anterior most lateral invagination sites of each hemisegment. (B) Light micrograph of the opisthosoma of a 200 hour embryo labeled with an antibody against HRP. The arrows indicate the anterior most lateral invagination sites of each hemisegment. The arrowhead points to a lateral axon fascicle. *o1* to *o5*, opisthosomal segments 1 to 5. Scale bars: 150 μm.

females of *Pholcus phalangioides* and stained embryos that were in the process of inversion with phalloidin-rhodamine. At the stage analysed, 30 to 32 invagination sites can be detected in the prosomal hemisegments and about 25 in the first to fifth opisthosomal hemisegments of *Pholcus* embryos (Fig. 6). The number of invagination sites corresponds to those of a *Cupiennius salei* embryo 190 hours after egg laying. The invagination sites in the prosoma of *Pholcus* are arranged in seven rows of four to five invagination sites each, as in *Cupiennius*. Six rows of invagination sites can be counted in each of the first to fifth opisthosomal hemisegments. The same pattern can be seen at a comparable stage in *Cupiennius* (Fig. 6C,D). These data show that the pattern of invagination appears to be conserved among the Araneae.

Mitotic divisions during neurogenesis

It is suggested in the literature that in arachnids neurogenesis occurs by a generalized inward proliferation of neuroectodermal cells to produce paired segmental thickenings (Anderson, 1973). Furthermore, it has been suggested that in some arachnids, the Amblypygids and the Araneids, each neuromere is formed by a number of invaginations. Cells divide rapidly and form small clusters that invaginate. They continue to proliferate when the innermost cells are differentiating into neurones (Barth, 1985). These descriptions suggest that there is a connection between invagination and cell division. To reinvestigate these statements and to see whether the same situation can be found in the ventral neuroectoderm of *Cupiennius*, embryos were double stained with phalloidin-rhodamine and the anti-phospho-histone 3 (PH3) antibody, which marks mitotic cells. The pattern of mitotic cells was analysed from the beginning of invagination to the end of inversion. At 130 hours, when the first 5 to 8 invagination sites are visible, only single stained cells can be detected in the ventral neuroectoderm (Fig. 7A,C). The same pattern can be

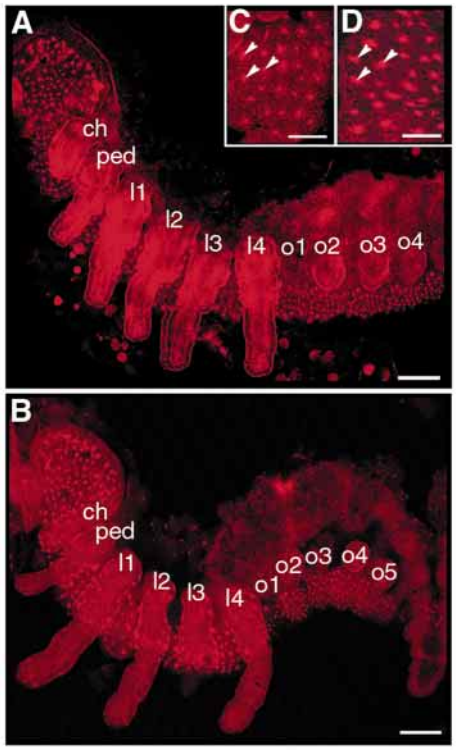

Fig. 6. (A-D) Comparison of invagination sites in *Cupiennius salei* and *Pholcus phalangioides*. Both embryos were stained with phalloidin-rhodamine. (A) Confocal micrograph of a flat preparation of a *Cupiennius salei* embryo at 200 hours. The ventral neuroectoderm has developed all 30 to 32 invagination sites. (B) Confocal micrograph of a flat preparation of a *Pholcus phalangioides* embryo. Despite the fact that the walking legs are longer than those of the *Cupiennius* embryo shown in A, the *Pholcus* embryo seems to be somewhat younger because the opisthosoma has not developed all invagination sites yet. (C) Invagination sites of the third opisthosomal segment of *Cupiennius* at 190 hours. There are six rows of invagination sites at this stage. The arrowheads point to the two anterior most lateral invagination sites. (D) In the third opisthosomal hemisegment of a *Pholcus* embryo at a stage comparable with the *Cupiennius* embryo shown in C, six rows of invagination sites can also be counted. The arrowheads point to lateral invagination sites of the hemisegment, which are located at similar positions in *Cupiennius* and *Pholcus*. *Ch*, cheliceral segment; *ped*, pedipalpal segment; *l1* to *l4*, walking legs 1 to 4 (corresponding to prosomal segments 3 to 6); *o1* to *o5*, opisthosomal segments 1 to 5. Scale bars: 200 μm in A,B; 100 μm in C,D.

seen until 175 hours (Fig. 7B,D). However, 190 hours after egg laying, many cells undergo mitosis in the ventral neuroectoderm. The dividing cells are partially grouped together, whereas in some cases individual mitotic cells can still be seen (Fig. 7F). Some of the dividing cell clusters are located around invaginating cells (Fig. 7F, arrow). However, analysis of transverse sections revealed that there are no cell divisions within the groups of invaginating cells (Fig. 7J, arrows). In addition, mitotic activity seems to be restricted to the apical layer of the ventral neuroectoderm (Fig. 7I, arrow) with the exception of a few cells (Fig. 7I, arrowhead). Furthermore, in all cases analysed, the mitotic spindles were orientated parallel to the apical surface (Fig. 7E, arrow). This means that the daughter cells are not automatically pushed into a more basal position after division, rather they remain in the most apical layer of the ventral neuroectoderm. After formation of all invagination sites, only a few labelled cells can be detected in the neuroectoderm (Fig. 7G). A new wave of mitosis arises during the decrease of the invagination sites (Fig. 7H). As before, both single mitotic cells and groups of cells are labelled. At that time there is also no mitosis within the groups of invaginating cells as could be shown by analyses of transverse optical sections (Fig. 7J, arrows). Cells that have already invaginated do not divide either (Fig. 7J, asterisks), rather they start to form a neuropil by growing axons (see Fig. 4H, arrow). These data show that there is no obvious correlation between cell proliferation and invagination in the ventral neuroectoderm of *Cupiennius salei*. In addition, it could not be confirmed that the invaginated cells continue to proliferate while more basally located cells start to differentiate into neurones.

Isolation of CsASH1 and CsASH2 cDNA

The sequential formation and the regular arrangement of the invagination sites in the ventral neuroectoderm of *Cupiennius* suggests that there must be a mechanism that confers neural potential on groups of neuroectodermal cells at a certain time point. In *Drosophila* the *achaete-scute* complex (ASC) is part of a cascade of genes that are responsible for the establishment of neural precursors (Campuzano and Modolell, 1992; Ghysen and Dambly-Chaudière, 1988; Jan and Jan, 1994; Skeath and Carroll, 1994). The ASC genes belong to a family of genes encoding basic-helix-loop-helix (bHLH) transcriptional regulators, which are important regulatory components of transcriptional networks of many developmental processes (Murre et al., 1989). These proteins share two common motifs: a domain of basic amino-acids required for DNA binding and a helix-loop-helix domain involved in protein dimerization (Murre et al., 1994).

As proneural genes have not been found in arthropods other than insects, we tried to identify these genes in the spider using PCR and degenerate oligonucleotide primers that correspond to sequences conserved between the *Drosophila* ASC genes *achaete* (*ac*), *scute* (*sc*) and *lethal of scute* (*l(1)sc*), five invertebrate (Villares and Cabrera, 1987; Martin-Bermudo et al., 1993; Takano, 1998; Galant et al., 1998; Krause et al., 1997; Grens et al., 1995) and three vertebrate ASC homologues (Allende and Weinberg, 1994; Jasoni et al., 1994; Tanaka et al., 1999). PCR of cDNA from embryonic stages of *Cupiennius* yielded several weak bands of the expected size (114 to 138 bp). Amplification of these PCR products using nested PCR resulted in a 120 bp band that was cloned into pZero. Forty-eight clones with appropriately sized inserts were randomly selected and sequenced. 12 of the 48 clones had inserts, each of which contained seven conserved amino acids of the first helix and the loop region of the *achaete-scute* family at the deduced amino acid level, while others were unrelated. The PCR fragments fall into two groups that could be distinguished by differences at five amino acid positions. In order to isolate the whole sequences, we performed 5′ and 3′RACE (rapid amplification of cDNA ends) using transcript specific primers that were directed against the two different PCR fragments. With this method, we identified two proneural genes in the spider: *Cupiennius salei achaete-scute* homolog 1 (*CsASH1*) and 2 (*CsASH2*).

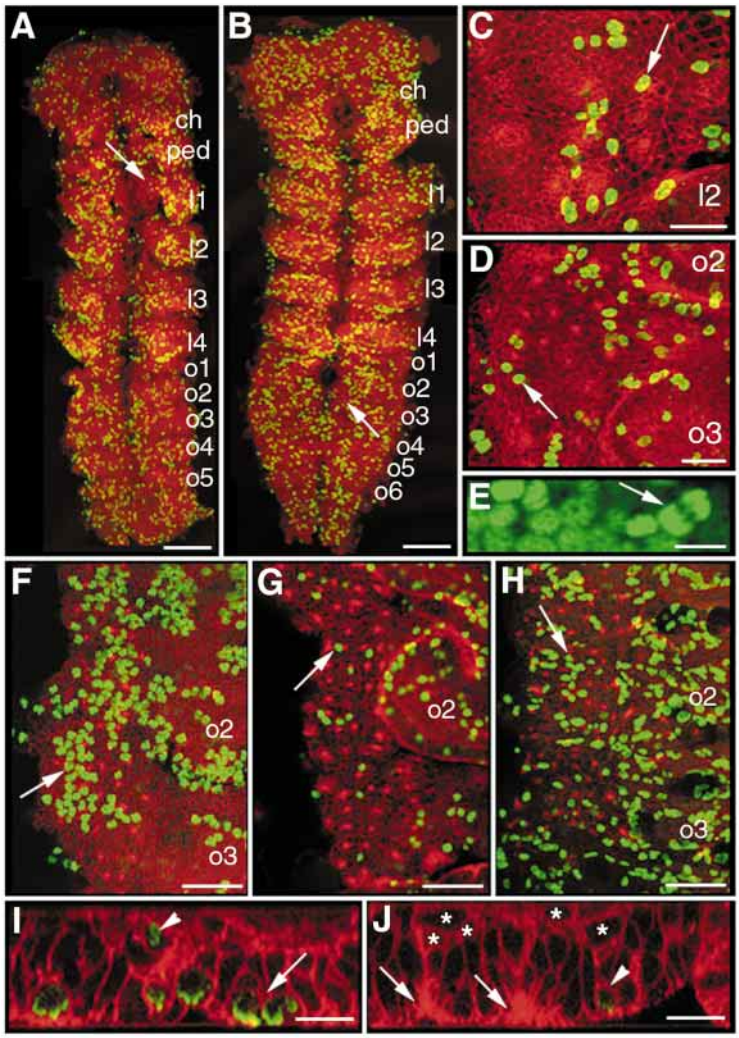

Fig. 7. (A-J) Mitotic divisions during neurogenesis. (A-D,F-H) Confocal micrographs of flat preparations of embryos stained with phalloidin-rhodamine and anti-PH3; anterior is towards the top. (A) At 130 hours, when the first five to eight invagination sites (arrow) are visible, only single cells are marked by anti-PH3. (B) The same pattern can be seen until 180 hours after egg laying. (C,D) Higher magnifications of A,B, respectively. The arrows point to single cells marked by anti-PH3. (E) Horizontal optical section of the apical region of the ventral neuroectoderm. Cell nuclei are stained with YOYO. The cells divide perpendicular to the surface. The arrow points to the separated chromosomes of a dividing cell. (F) At 190 hours, when most of the invagination sites have already formed, groups of labelled cells can be seen in the ventral neuroectoderm (arrow). (G) At 200 hours, when all invagination sites have formed, only single labelled cell can be detected (arrow). During decrease of the invagination sites at 220 to 240 hours, groups of labelled cells are visible again (arrow). (I) Transverse optical section of the ventral neuroectoderm of a 190 hour embryo. Cell division is restricted to the apical layer of the ventral neuroectoderm (arrow) with the exception of a few cells (arrowhead). (J) Transverse optical section of the ventral neuroectoderm of a 220 hour embryo. The invaginated cells do not divide (asterisks). Dividing cells can be seen only in the apical layer (arrowhead). The arrows point to invagination sites. *Ch*, cheliceral segment; *ped*, pedipalpal segment; *l1* to *l4*, walking legs 1 to 4 (corresponding to prosomal segments 3 to 6); *o1* to *o6*, opisthosomal segments 1 to 6. Scale bars: 200 μm in A,B; 40 μm in C; 50 μm in D; 10 μm in E; 100 μm in F-H; 20 μm in I,J.

The 840 bp sequence obtained for *CsASH1* encodes a deduced protein of 197 amino acids. Comparison of the amino acid sequence of the bHLH domain shows that *CsASH1* has 82% identity to the chicken *ac-sc* homolog *CASH1* and about 70% identity to the *Drosophila* ASC members *ac*, *sc* and *l(1)sc*. (Fig. 8A). The 1056 bp sequence obtained for *CsASH2* encodes a deduced protein of 203 amino acids which is 82% identical to the frog *ac-sc* homologue *XASH1* over the region encoding the bHLH domain and about 70% identical to the gene products of the *Drosophila* ASC members *ac*, *sc* and *l(1)sc* over the same region (Fig. 8B). In common with the vertebrate *ac-sc* homologue proteins, the loop region of the bHLH domain of both identified genes is shorter than equivalent regions of the *Drosophila* ASC proteins. Both genes are clearly *ac-sc* homologues, as comparison of their bHLH domains with that of other families of bHLH proteins shows a much lower degree of amino acid sequence identity (data not shown). Outside of the bHLH domain *CsASH1*and *CsASH2* diverge from all other *ac-sc* homologues. Comparison of the identified genes with each other revealed that there are differences at nine amino acid positions over the region of the bHLH domain, which corresponds to an identity of 82%. Apart of the first three amino acids following the translation initiation sites (MASL) the deduced amino acid sequences of CsASH1 (EMBL, AJ309490) and CsASH2 (EMBL, AJ309491) are distinct from each other outside the bHLH region.

CsASH1 expression during neurogenesis

CsASH1-expressing cells were localized by whole-mount in situ hybridisation using digoxygenin (DIG)-labelled riboprobes. *CsASH1* transcripts are first detected at 100 to 120 hours after egg laying in the cephalic lobe and the ventral neuroectoderm. At that time point, no invagination sites can be seen in the neuroectoderm (see Fig. 4A). Two medial and two lateral cell clusters express the gene in the cephalic lobe (Fig. 9A). In the ventral neuroectoderm *CsASH1* is expressed in groups of cells that are located in the anterior most lateral region of the prosomal hemisegments and the first opisthosomal hemisegment (Fig. 9F,K). The boundaries of the *CsASH1* domains are not sharp and there is also heterogeneity in the expression levels within these domains (Fig. 9P). 130 hours after egg laying, the first five to eight invagination sites arise in the prosomal hemisegments and the first opisthosomal hemisegment exactly in the region of *CsASH1* expression (see Fig. 4B). At that time point, the expression domain has extended posteriorly in all prosomal hemisegments (Fig. 9G,Q). In addition, in the anterior region of each prosomal hemisegment cells which are located medially to the former

A

	basic	helix	loop	helix	
CsASH1	RRNERERNRVRQVNLGFATLRQHVP	........N	........RSKKMSKVETLRSAVQYIRQL		
CASH-1	----------	KL---------	E---.....-G.....	AAN----------	E---A- 82%
Achaete	----------	K---N--SQ----	I-AAVIADLS-GRRGIGPGAN--L---	S--KM--E---R- 69%	
Scute	---A------	K---NS--R----	I-QSIITDLTKG..G.GRGPH--I---	D---I--E---S- 67%	
L´sc	---A------	K---N--VN----	L-QTVVNSLS-G....GRGS---L---	D---I--E---G- 71%	

B

CsASH2	RRNERERNRVRLVNLGFANLRQHVP	........NS	.....SKNKKMSKVDTLRSAVEYIKQL		
XASH1	----------	K-------T--E---	.....-G.....	AA-------	E---------RA- 82%
Achaete	---A------	KQ--N--SQ----	I-AAVIADLS-GRRGIGPGA---	L---	S--KM-----RR- 67%
Scute	---A------	K---NS--R----	I-QSIITDLTKG..G.GRGPH--I-	-------	I-----RS- 69%
L´sc	---A------	KQ--N--V-----	L-QTVVNSLS-G....GRGSS--L-	-------	I-----RG- 71%

Fig. 8. (A,B) Deduced amino acid sequence of the bHLH domain of *CsASH1* (A) and *CsASH2* (B), and their relationship to other bHLH proteins. The bHLH domain is indicated. The alignment compares the basic domain and the two helices of CsASH1 and CsASH2 with three members of the ASC family of *Drosophila* and with the genes from other species that show the highest identity to the proneural genes of the spider. CsASH1 shows the highest identity to the chicken proneural protein CASH-1, whereas CsASH2 is highly identical to the proneural protein XASH1 of *Xenopus*. The loop, which varies in length and in sequence, is not included in the percent amino acid identity calculation. Dashes indicate amino acid identity with *CsASH1* and *CsASH2*, and dots indicate gaps in the protein alignment. GenBank Accession Numbers, AJ309490 and AJ309491.

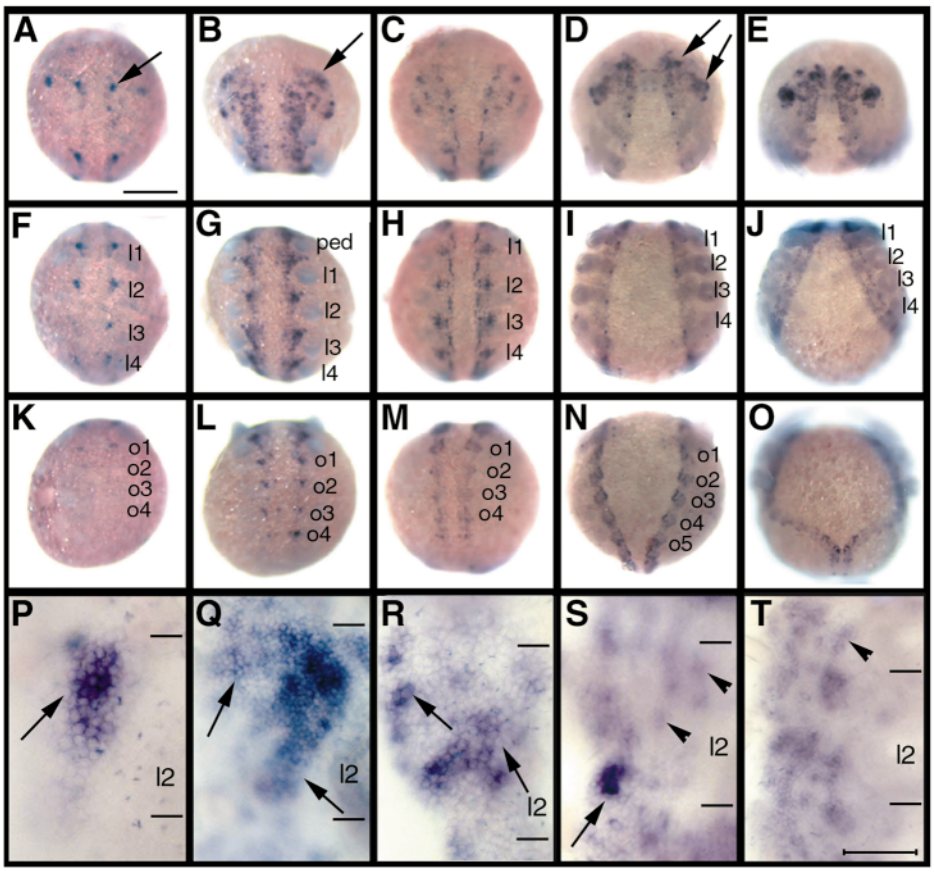

Fig. 9. (A-T) Expression pattern of *CsASH1*. (A-O) Whole mounts; anterior is towards the top. (A-E) Cephalic lobe; (F-J) prosoma; (K-O) opisthosoma; (P-T) flat preparations of the fourth prosomal segments; anterior is towards the top, the black lines indicate the segmental borders. (A,F,K) At 120 hours, *CsASH1* staining is visible in the cephalic lobe (arrow in A), in the anterior-most lateral regions of all hemisegments of the prosoma (F,P) and in the first opisthosomal segment (K). (B,G,L) At 130 hours, *CsASH1* staining has extended posteriorly and medially in the prosoma (G, arrows in Q). The staining in the anterior-most lateral region that was visible in the prosoma and the first opisthosomal hemisegments before can now be seen in opisthosomal segments o2 to o4 (L). Additional expression domains can be also detected in the cephalic lobe. (C,H,M) At 160 hours, *CsASH1* expression has decreased in the cephalic lobe (C) and in the second to sixth opisthosomal hemisegments (M). The RNA is now expressed in a lateral and a medial stripe of cells in the prosomal and the first opisthosomal hemisegments (H,M,arrows in R). (D,I,N) At 180 hours, new expression domains can be seen in the cephalic lobe (D, arrows). The former *CsASH1* expression has disappeared in the prosoma and the first opisthosomal segment and a new medial expression domain is visible (I, arrow in S). The expression pattern in the opisthosoma reflects that seen before in the prosoma (N). *CsASH1* is expressed weakly in the invaginating neuroectodermal cells (S, arrowheads). (E,J,O) At 220 hours, the medial expression domain has disappeared with the exception of the last three segments of the opisthosoma (O). *CsASH1* is only weakly expressed in the invaginating cells (T, arrowhead). *ped*, pedipalpal segment; *l1* to *l4*, walking legs 1 to 4 (corresponding to prosomal segments 3 to 6); *o1* to *o5*, opisthosomal segments 1 to 5. Scale bar: 200 μm in A-O; 150 μm in P-T.

expression domain express *CsASH1* (Fig. 9Q). The medial expression can also be seen in the first opisthosomal segments. Additional expression domains can be also detected in the cephalic lobe (Fig. 9B, arrow). At about 150 hours, new invagination sites have formed in the posterior and the medial expression domains of *CsASH1* in the prosomal and the first opisthosomal segments (Fig. 4C, arrows). Whereas *CsASH1* expression decreases in the prosoma and the first opisthosomal segment at that time, it has extended posteriorly and medially in the second to fourth opisthosomal hemisegments (data not shown). At 160 hours, expression of *CsASH1* has also decreased in the cephalic lobe and in the second to fourth opisthosomal segments (Fig. 9C,M), while it is now expressed in a lateral and a medial stripe of cells in the prosomal and the first opisthosomal hemisegments (Fig. 9H,M,R). New invagination sites arise in these lateral and medial regions at

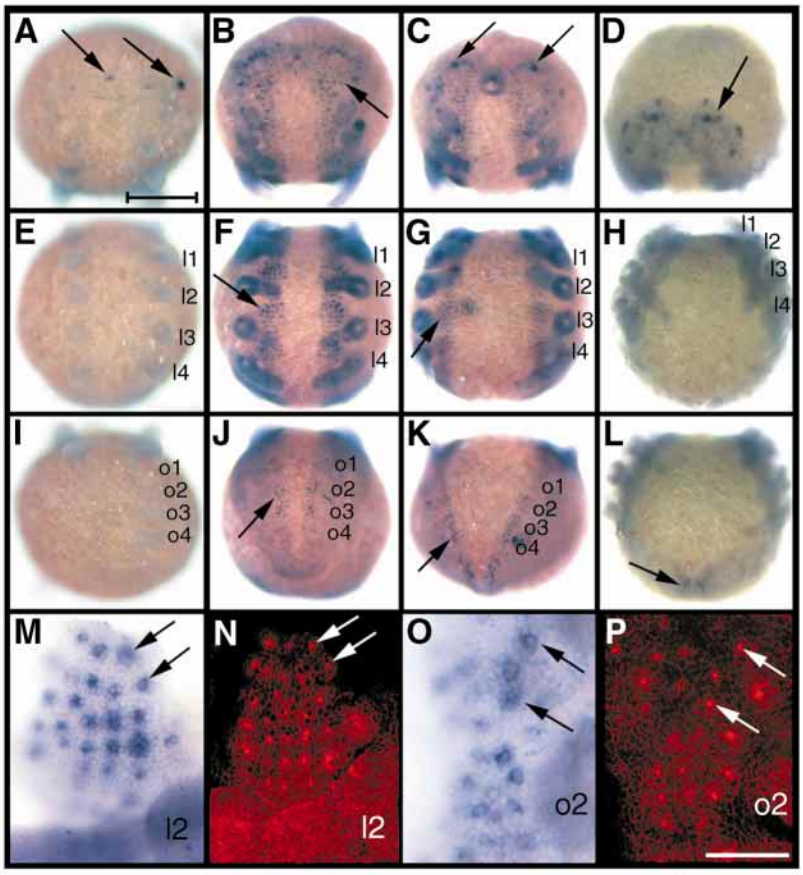

Fig. 10. (A-P) Expression pattern of *CsASH2*. (A-L) Whole mounts; anterior is towards the top. (A-D) Cephalic lobe; (E-H) prosoma; (I-L) opisthosoma; (M,N) flat preparations of the fourth prosomal hemisegment; ; (O,P) flat preparations of the second opisthosomal segment; anterior is towards the top. (A,E,I) At 130 hours, *CsASH2* expression can be seen only in two lateral and one median cell cluster in the cephalic lobe (A, arrows). (B,F,J) At 175 hours, *CsASH2* transcripts are visible in the invaginating neuroectodermal cells in the cephalic lobe (B, arrow) and in the ventral neuroectoderm (F,J, arrows). Expression has decreased in the two lateral cell clusters of the cephalic lobe, which were labelled before, while the median cell cluster still expresses the gene (B). (C,G,K) At 190 hours, *CsASH2* expression has decreased in the cephalic lobe, but now two lateral cell clusters show *CsASH2* expression (C, arrows). There is also a decrease of expression in the prosomal (G, arrow) and the first to second opisthosomal segments (K), while expression can still be seen in the remaining opisthosomal segments (K, arrow). (D,H,L) At 220 hours, additional regions of *CsASH2* expression can be detected in the cephalic lobe (D, arrow). In the ventral neuroectoderm, expression has disappeared in all segments with the exception of the last (H, arrows in L). (M) Higher magnification of the fourth prosomal hemisegment of an 175 hour embryo hybridised with the *CsASH2* probe. *CsASH2* is expressed in the invaginating cell groups (arrows). N shows the same region as M of a 175 hour embryo stained with phalloidin-rhodamine. The arrows point to the two anterior-most lateral invagination sites of the hemisegment (compare with arrows in M). (O) Higher magnification of the second opisthosomal hemisegment of an 175-hour embryo. Only five invaginating cell groups in the anterior-most lateral region show strong expression of *CsASH2*, whereas the remaining invaginating cells express the gene only weakly at this time. P shows the same region as in O of an embryo stained with phalloidin-rhodamine. The arrows point to two invagination sites, which show strong expression in O (arrows). *l1* to *l4*, walking legs 1 to 4 (corresponding to prosomal segments 3 to 6); *o1* to *o4*, opisthosomal segments 1 to 5. Scale bar: 200 μm in A-L; 100 μm in M-P.

about 175 hours (see Fig. 4D). At 180 hours the former *CsASH1* expression domains have disappeared in the prosoma and the first opisthosomal segments and a new medial expression domain can be detected (Fig. 9I,S), whereas the expression pattern in the opisthosoma reflects that seen before in the prosoma. In addition, *CsASH1* is expressed weakly in the invaginating neuroectodermal cells (Fig. 9S, arrowheads). New expression domains can also be seen in the cephalic lobe (Fig. 9D, arrows) at that time. At about 190 hours, all 30 to 32 invagination sites per hemisegment have formed in the prosoma (see Fig. 4E). The medial *CsASH1* expression has disappeared in the prosoma, whereas the expression pattern in the opisthosoma again reflects that seen before in the prosoma (data not shown). About 10 hours later, all 30 to 32 invagination sites can also be seen in the opisthosoma. The medial domain has disappeared from the opisthosoma, with the exception of the last three segments. In the remaining segments *CsASH1* is weakly expressed in the invaginating cells (Fig. 9T, arrowhead). The cephalic lobe still shows a strong expression in the same regions as before (Fig. 9E).

At about 200 hours, *CsASH1* also appears to be expressed in the PNS. The spider has a lot of mechanoreceptors, the most common of which is the hair sensillum. It appears as a simple tactile hair or as a more complex filiform hair (a trichobothrium). Although tactile hairs are distributed over the entire body surface, the trichobothria occur only on the extremities. They are much less numerous than the common tactile hairs, and are arranged in straight lines or in small clusters on certain leg segments (Foelix, 1996). *CsASH1* is expressed in four clusters of cells each in the pedipalps and the legs (see Fig. 11A). One cluster is located at the base of the extremity, one at the base of the second proximal segment and two on the most distal segment (see Fig. 11A, arrows). These cluster can also be seen by staining embryos with phalloidin (see Fig. 3A,B, arrowheads) or the anti-HRP antibody (see Figs 13A,D, 15A, arrows). At 220 hours, expression in the PNS disappears.

These data show that *CsASH1* is expressed in the ventral neuroectoderm in all regions of neurogenesis before invagination of neuroectodermal cells and in addition prior to formation of sensory organs in the PNS.

CsASH2 expression during neurogenesis

CsASH2-expressing cells were detected in the same way as *CsASH1* transcripts, by whole-mount in situ hybridisation using digoxigenin (DIG)-labelled riboprobes. *CsASH2* is expressed later in neurogenesis than *CsASH1*. Transcripts are first detected at 130 hours in two lateral and one median cell cluster in the cephalic lobe (Fig. 10A, arrows). At that time, the first invagination sites can already be seen in the cephalic

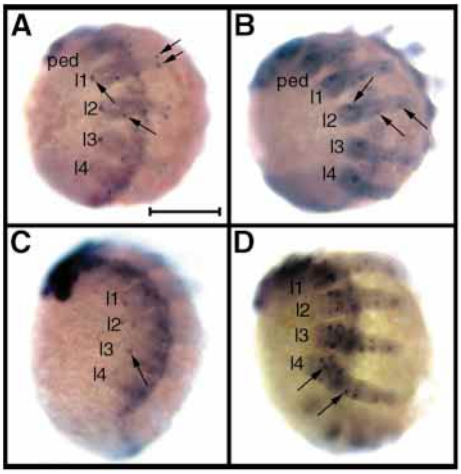

Fig. 11. (A-D) Expression pattern of *CsASH1* and *CsASH2* in the PNS. Whole mounts; anterior is towards the top. (A) At 200 hours, *CsASH1* is expressed in four clusters of cells each in the pedipalps and the legs (arrows). (B) At the same time, CsASH2 is also expressed in the PNS in non overlapping cell clusters (arrows). (C) At 220 hours, CsASH1 expression in the PNS disappears. There is only a weak staining left at the base of the extremities. (D) At the same time, *CsASH2* is expressed in 11 new cell clusters (arrows). Scale bar: 200 μm.

lobe and the prosomal and first opisthosomal hemisegments (see Fig. 4B). In the ventral neuroectoderm *CsASH2*-expressing cells are not labelled until 175 hours. Expression can be detected only in the invaginating neuroectodermal cells (Fig. 10F,J,M). In the cephalic lobe, *CsASH2* is also expressed in invaginating cells (Fig. 10B, arrow) and in addition in one median cell cluster (Fig. 10B, arrowhead). Expression has decreased in the two lateral cell clusters of the cephalic lobe labelled before. *CsASH2* is strongly expressed in all 22 to 25 invaginating cell groups of each prosomal hemisegment (Fig. 10F,M,N). In the first to fifth opisthosomal hemisegments, which have developed 16 to 17 invagination sites at that time (Fig. 10P), five to eight invaginating cell groups in the anterior most lateral region show a strong expression of *CsASH2* (Fig. 10O), whereas the remaining invaginating cells express the gene only weakly at that time. About 10 hours later, the gene is expressed strongly in all invaginating cells of the opisthosomal segments (data not shown). At 190 hours, *CsASH2* expression has already decreased in the prosomal and the first to second opisthosomal segments, while expression can still be seen in the remaining opisthosomal segments. There is also a decrease of expression in the invaginating cells of the cephalic lobe, but now two lateral cell clusters show *CsASH2* expression (Fig. 10C, arrows). At 220 hours, additional regions of *CsASH2* expression can be detected in the cephalic lobe, whereas in the ventral neuroectoderm the expression has disappeared in all segments with the exception of the last one (Fig. 10H,L, arrow).

At about 200 hours *CsASH2* is expressed in three clusters of cells in the PNS (Fig. 11B). One cluster is located in the first proximal segment of the extremity, one in the second and one in the most distal segment (Fig. 11B, arrows). *CsASH2* expression in the extremities does not overlap with those of *CsASH1* (compare Fig.11A with 11B). 20 hours later, when *CsASH1* expression has already decreased in the PNS, about 11 new cell clusters each express *CsASH2* in the extremities (Fig. 11D, arrows).

These data show that *CsASH2* is transiently expressed in neuroectodermal cells during the process of invagination. In addition, the gene is partially expressed in patches of cells in the cephalic lobe, similar to *CsASH1*, but not in overlapping regions (compare Fig. 9A-E with Fig. 10A-D).

Analysis of CsASH1 function during neurogenesis

Recently, the use of double-stranded RNA (dsRNA) to interfere with gene function in *Caenorhabditis elegans* (Fire et al., 1998) has been successfully extended to *Drosophila* (Kennerdell and Carthew, 1998; Misquitta and Paterson, 1999) and other insects (Brown et al., 1999; Hughes and Kaufman, 2000). The so-called RNA-mediated interference (RNAi) technique has also been successfully established for the spider *Cupiennius salei* (Schoppmeier and Damen, 2001). To analyse the function of *CsASH1* during neurogenesis, we injected dsRNA corresponding to the basic domain and the first helix of the bHLH domain, and the complete upstream part of the gene. Injected spider embryos were cultivated (see Materials and Methods) until about 220 hours after egg laying. The resulting phenotypes were investigated by staining 10% of the embryos with phalloidin-rhodamine and the remaining 90% with HRP antibody. 60.5% of the embryos which had no developmental arrest after injection showed a specific phenotype (Table 1). Although embryos injected as a control with dsRNA corresponding to a portion of the green fluorescent protein (GFP) exhibit the normal number of invagination sites in the ventral neuroectoderm (Figs 12A, 13A,D), embryos injected with *CsASH1* dsRNA are missing the invagination sites to different degrees. 42% of the affected embryos have no invagination sites either in the prosoma or the opisthosoma (Fig. 13E), 37% show a reduction of invagination sites in individual segments or over the whole region of the neuroectoderm (Figs 12B, 13B,F). 11.5% of the embryos show asymmetric effects: only one half of the germ band is affected (Fig. 13C). In 9% of the embryos that show a specific phenotype, no invagination sites can be detected at all (data not shown). Analyses of transverse optical sections in the LSM revealed that in regions of the ventral neuroectoderm where no invagination sites can be detected the morphology of the neuroectodermal cells is disturbed (Fig. 12D). The typical columnar shape of neuroectodermal cells is lost; instead, the cells have a rounded appearance (Fig. 12D). In segments where only a reduced number of invagination sites develop, the process of invagination seems to be blocked (Fig. 12E). Although in control injected embryos a basal layer of neuroectodermal cells that have already invaginated can be detected (Fig. 12C, bracket), there seems to be no invagination in the affected segments, as no second cell layer is visible (Fig. 12E).

These data show that *CsASH1* has an early function in neurogenesis. The activity of the gene seems to be required both for the formation of the invagination sites and the process of invagination itself.

Analysis of CsASH2 function during neurogenesis

To analyse the function of *CsASH2* during neurogenesis, we injected dsRNA corresponding to the first helix, the loop and the second helix of the bHLH domain, and the complete

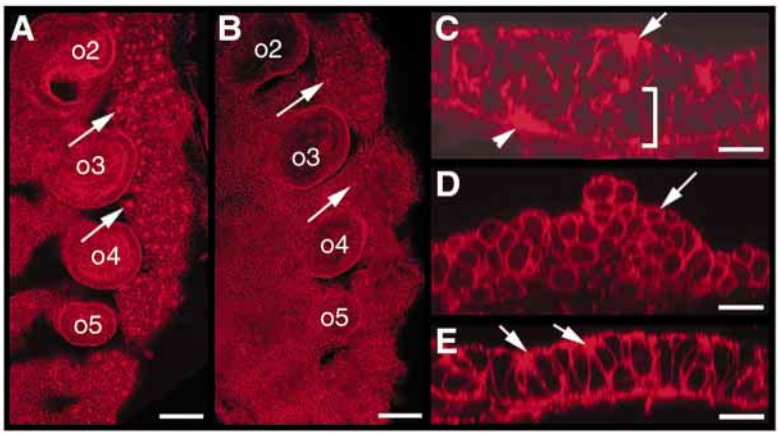

Fig. 12. (A-E) Phenotypic analysis of embryos stained with phalloidin-rhodamine after injection of *CsASH1* dsRNA. (A) Opisthosoma of an embryo injected with *GFP* dsRNA as a control. The embryo shows the normal number of invagination sites in the ventral neuroectoderm. The arrows point to the anterior-most lateral invagination sites. (B) Opisthosoma of an embryo injected with *CsASH1* dsRNA. No invagination sites can be detected in the ventral neuroectoderm, with the exception of two lateral invagination sites (arrows). (C) Transverse optical section of an embryo injected with *GFP* dsRNA as a control; apical is towards the top. The invagination sites have decreased (arrow) and a second layer (bracket) of invaginated cells has formed. A developing neuropil is visible basally (arrowhead). (D,E) Transverse optical section of embryos injected with *CsASH1* dsRNA; apical is towards the top. (D) The morphology of the neuroectodermal cells is disturbed, the cells have a rounded appearance (arrow). (E) In this segment, where only a reduced number of invagination sites has developed (arrows), the process of invagination seems to be blocked: no second cell layer is visible. *o2* to *o5*, opisthosomal segments 1 to 5. Scale bars: 100 μm in A,B; 20 μm in C-E.

Table 1. Summary of RNAi experiments

(A) *GFP* dsRNA was injected as a control*

	Injected	Developed	Specific phenotype	Nonspecific phenotype	Normal
GFP	198 (100%)	132 (67%)	0 (0%)	62 (47%)	70 (53%)
CsASH1	593 (100%)	438 (73%)	265 (60.5%)	150 (34%)	23 (5%)
CsASH2	703 (100%)	480 (68%)	303 (63%)	160 (33%)	17 (3.5%)

(B) Embryos which show a specific phenotype can be grouped into four clusters‡

	Asymmetric effects	Reduction (single segments)	Prosoma or opisthosoma	Whole germ band
Invagination sites affected – *CsASH1* injection	11.5%	37%	42%	9%
Neuropil affected – *CsASH2* injection	10%	82%	8%	0%

*Injected, the number of embryos that were injected either with *GFP* or *CsASH1* and *CsASH2* dsRNA; developed, the number of embryos that developed further up to the time where they were analysed (about 200 hours); specific phenotype, the number of embryos exhibiting a specific phenotype after injection that can be traced back to loss of function of the corresponding gene; nonspecific phenotype, the number of embryos exhibiting nonspecific defects, such as reduced or undeveloped cephalic lobe, reduced or undeveloped prosoma or opisthosoma; normal, embryos that show the same phenotype as untreated embryos

‡Asymmetric effects, only on half of the germ band is affected; reduction (single segments), reduction of invagination sites (*CsASH1*) or neuropil (*CsASH2*) over the whole germ band, in individual segments, or complete absence of the invagination sites (*CsASH1*) or the neuropil (*CsASH2*) in individual segments; prosoma or opisthosoma, invagination sites (*CsASH1*) or neuropil (*CsASH2*) are missing either in the prosoma or the opisthosoma; whole germ band, no invagination sites (*CsASH1*) or neuropil (*CsASH2*) can be detected in the ventral neuroectoderm.

downstream part of the gene. The embryos were treated and analysed in the same way as has been described above for *CsASH1* dsRNA injection. Injection of *CsASH2* dsRNA did not affect the formation of the invagination sites nor the process of invagination itself (Fig. 14B). Rather, the differentiation of the invaginated neural precursor cells seems to be disturbed, as in the affected embryos, a reduction of the neuropil could be detected in individual or several ventral neuromeres (Fig. 14E,F). In the affected neuromeres the axon fascicle, which connects the individual neuropils is missing (Fig. 14C,D, arrowhead). In 8% of the embryos that have a specific phenotype the neuropil is either completely absent in the prosoma or in the opisthosoma. 10% of the embryos that have reduced neuropils show an asymmetric effect, as only one half of the germ band is affected. Despite the fact that the anti-HRP staining can be detected earlier during neurogenesis than *CsASH2*, HRP staining is reduced or partially absent in the affected embryos (Fig. 15B,D).

These data show that *CsASH2* has a later function in neurogenesis than *CsASH1*. The gene seems to be involved in the differentiation of the neural precursor cells.

DISCUSSION

Sequential formation and regular pattern of invagination sites

Our results on the process of neurogenesis in the spider *Cupiennius salei* contrast partially to the description of arachnid neurogenesis by other authors (Anderson, 1973; Barth, 1985). These authors report that arachnid neurogenesis occurs by a generalized inward proliferation of neuroectodermal cells to produce paired thickenings. In some arachnids, the Amblypygids and the Araneids, each neuromere is supposed to be formed by a number of invaginations. In this case, cells also divide rapidly and form small cell clusters that invaginate. This kind of neurogenesis implies that cell proliferation controls the process of invagination. This is in

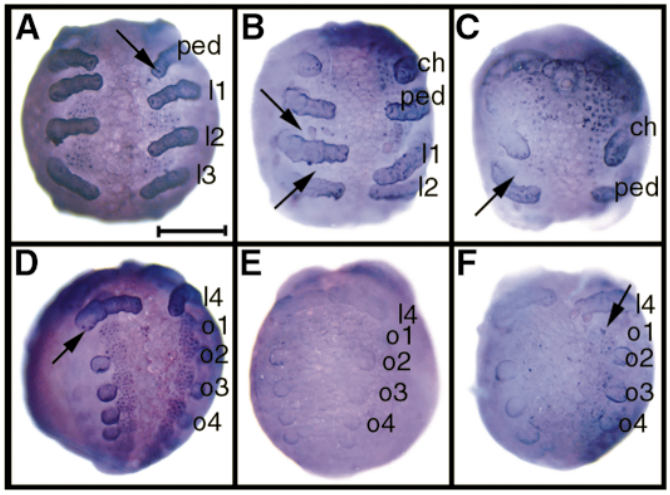

Fig. 13. (A-D) Phenotypic analysis of embryos stained with an antibody against HRP after injection of *CsASH1* dsRNA; anterior is towards the top. (A,D) The prosoma and opisthosoma, respectively, of an embryo injected with *GFP* dsRNA as a control. The embryo shows the normal HRP staining. The arrows point to staining in the PNS. (B) Prosoma of an embryo injected with *CsASH1* dsRNA. Several segments of the prosoma show no HRP staining (arrows). (C) Prosoma of an embryo injected with *CsASH1* dsRNA. This embryo shows an asymmetric effect: only one half of the germ band shows a reduced staining. (E) Opisthosoma of an embryo injected with *CsASH1* dsRNA. All invagination sites are missing in the opisthosoma. (F) Opisthosoma of an embryo injected with *CsASH1* dsRNA. Invagination sites are reduced in the opisthosoma. Only the anterior-most lateral invagination sites are visible (arrow). *Ch*, cheliceral segment; *ped*, pedipalpal segment; *l1* to *l4*, walking legs 1 to 4 (corresponding to prosomal segments 3 to 6); *o1* to *o4*, opisthosomal segments 1 to 4. Scale bar: 200 μm.

contrast to the advanced mode of neurogenesis in the insect *Drosophila*. In *Drosophila* the neuroblasts do not perform their first postblastodermal division until delamination: whereas the nuclei of the cells remaining in the ventral neuroectoderm shift apically in preparation for mitosis, the nuclei of the presumptive neuroblasts remain basally and postpone mitosis (Hartenstein et al., 1994). Furthermore, the neuroblasts arise at certain time points and at stereotyped positions that are defined by the expression domains of the proneural genes. However, our results suggest that neurogenesis in the spider *Cupiennius* does not proceed as it is described in these reports. We show that neural precursor cells arise sequentially and at stereotyped positions in the ventral neuroectoderm of the spider. The first five to eight invagination sites are visible in the anterior most lateral region of the hemisegments shortly after formation of the limb buds. The next wave generates nine to 12 new invagination sites in a posterior region adjacent to the anterior domain and in the medial anterior most region of each hemisegment. Subsequently, five to eight invagination sites are formed in the lateral and medial regions of each hemisegment. Finally, seven to ten invagination sites are added between rows three and four, and at the posterior end of each hemisegment. Eventually there are seven rows of four to five invagination sites each in all hemisegment of the prosoma and the opisthosoma. Interestingly, the same general pattern of invagination sites can be detected in the distantly related spider *Pholcus phalangioides*. These data show that the invagination sites arise at stereotyped positions in the ventral neuroectoderm. In addition, the formation of the invagination sites takes place in four discrete waves. This mode of generation of neural cells shows similarities to neurogenesis in *Drosophila*. In *Drosophila*, the 25 neuroblasts per hemisegment are also generated sequentially (in five waves) and occur at stereotyped positions. The first two populations of neuroblasts are arranged in three longitudinal columns and four rows per hemisegment. This regular pattern is lost after delamination of the next population of neuroblasts, as the earlier neuroblasts are shifted into a more basal position (Goodman and Doe, 1993).

Although the formation of invagination sites in the spider occurs sequentially, comparable with the generation of neuroblasts in *Drosophila*, the process of invagination itself seems then to be restricted to a short period of time. Invagination sites in the spider are generated over a period of 3 days. The fact that all 30 to 32 invagination sites are visible simultaneously until shortly before the end of inversion implies that at least some of the cells of the invaginating cell groups are still connected to the apical surface. In line with these observations, transverse optical sections reveal that basal cell layers can not be detected until the third day of neurogenesis (see Fig. 12C, bracket). Only 1 day later, when the invagination sites disappear, a thick layer of invaginated neural cells is visible basally (see Fig. 4G). This observation suggests that the recruitment of neuroectodermal cells for the neural fate during early neurogenesis is separated from the final differentiation of those cells by several days. This contrasts with *Drosophila*, where each population of neuroblasts delaminates from the ventral neuroectoderm before the next wave of neuroblasts arises. In addition, the delaminated neuroblasts take up their final function by dividing to produce ganglion mother cells within a few minutes of segregation.

Mitotic divisions in the ventral neuroectoderm

As described above, earlier morphological studies have in general suggested that the process of invagination is tightly connected to cell proliferation. In addition, the authors claim that the invaginated cells continue to divide when the innermost cells are differentiating into neurones and growing axons (Barth, 1985). We addressed this issue by staining *Cupiennius* embryos with the anti-PH3 antibody, which labels mitotic cells. Analyses of the mitotic divisions in the ventral neuroectoderm revealed that during most of neurogenesis, only single cells divide. Clusters of proliferating cells cannot be detected until the fourth wave of formation of invagination sites. At that time point, groups of mitotic cells are located in regions where new invagination sites arise (see Fig. 7F, arrow). However, proliferating cell groups can also be detected in regions of the hemisegments where all invagination sites have already formed. These data show that in contrast to earlier studies on other arachnids there is no obvious connection between cell proliferation and invagination in *Cupiennius*.

Furthermore, our results do not support earlier observations in other arachnids, which show that the neuroectodermal cells keep dividing after invagination. Analyses of transverse optical sections revealed that, throughout neurogenesis, almost all cell divisions occur in the apical layer of the ventral neuroectoderm. Mitotic divisions could not be detected within the groups of invaginating cells and only in rare cases were

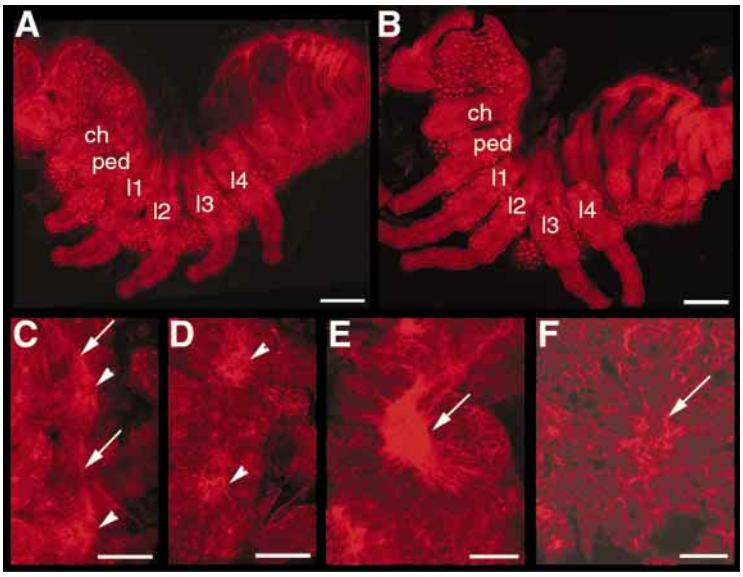

Fig. 14. (A-F) Phenotypic analysis of embryos stained with phalloidin-rhodamine after injection of *CsASH2* dsRNA. (A) Flat preparation of an embryo injected with *GFP* dsRNA as a control. The embryo shows the normal number of invagination sites in the ventral neuroectoderm and the cephalic lobe; anterior is towards the left. (B) Flat preparation of an embryo injected with *CsASH2* dsRNA. The embryo shows the normal number of invagination sites in the ventral neuroectoderm and the cephalic lobe; anterior is towards the left. (C) Flat preparation of the second and third prosomal hemisegements of an embryo injected with *GFP* dsRNA as a control. The arrowheads point to the developing neuropils of the hemisegments, which are connected by an axon fascicle (arrows). (D) Flat preparation of the second and third prosomal hemisegments of an embryo injected with *CsASH2* dsRNA. The arrowheads point to the reduced neuropils. The axon fascicle connecting the neuropils in the control embryos are missing in *CsASH2*-injected embryos. (E) Higher magnification of the third prosomal segment of an embryo injected with GFP. The arrow points to the neuropil. (F) Higher magnification of the third prosomal segment of an embryo injected with *CsASH2* dsRNA. The neuropil is strongly reduced (arrow). *Ch*, cheliceral segment; *ped*, pedipalpal segment; *l1* to *l4*, walking legs 1 to 4 (corresponding to prosomal segments 3 to 6). Scale bars: 200 μm in A,B; 150 μm in C,D; 40 μm in E,F.

divisions seen in basally located cells that had already invaginated. These observations suggest that the basal cells are neuronal cells that differentiate further and start growing axons immediately after invagination. The fact that the neuronal anti-HRP antibody stains the invagination sites very early during neurogenesis confirms the assumption that the invaginating cells are neuronal cells that already express neuronal antigens.

Another interesting aspect of the analyses of mitotic divisions is that there seem to be no neural precursor cells with the characteristics of insect neuroblasts in the ventral neuroectoderm of the spider. The apically located proliferating cells in the ventral neuroectoderm of the spider divide perpendicular to the apical surface. This means that the daughter cells remain in the same cell layer and do not automatically shift basally after division. The fact that there is also almost no cell division in the basal layers implies that neural precursor cells that divide in a stem cell-like mode are not present in the spider. However, it is possible that at least in one case neuroblasts that divide in a stem cell-like mode are present in a chelicerate. In the pantopod *Callipallene emaciata* Winter (Winter, 1980) describes spindle-shaped cells that invaginate and divide to produce daughter cells that are arranged in radial rows. As the relationship of the pantopods to the remaining chelicerates is still being discussed, we do not know how these data fit into our findings.

As was mentioned above, neuroblasts that share many of the characteristics of insect neuroblasts could be detected in crustaceans. They divide asymmetrically with a spindle perpendicular to the surface. The larger basal daughter cell divides again with the same spindle orientation. Thus a column of ganglion mother cells is formed. In contrast to insects, crustacean neuroblasts do not delaminate from the apical neuroectodermal cell layer before or during their divisions. In addition, at least some crustacean neuroblasts can give rise to epidermal cells after they begun to bud off ganglion mother cells (Dohle and Scholz, 1988).

Considering this, one can speculate that the generation of

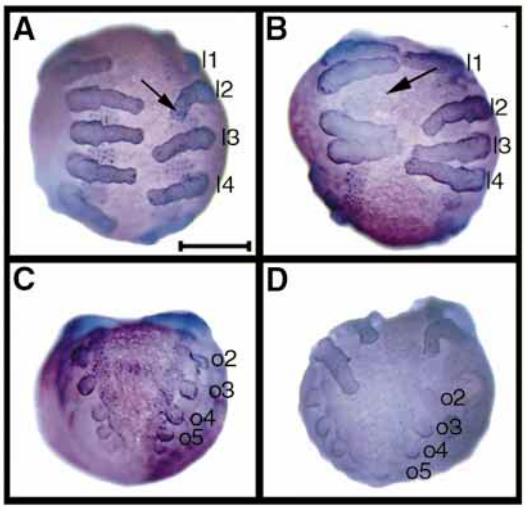

Fig. 15. (A-D) Phenotypic analysis of embryos stained against HRP after injection of *CsASH2* dsRNA; anterior is towards the top. (A,C) The prosoma and opisthosoma, respectively, of an embryo injected with *GFP* dsRNA as a control. The embryo shows the normal HRP staining. The arrow indicates staining in the PNS. (B) Prosoma of an embryo injected with *CsASH2* dsRNA. The embryo shows a reduced HRP staining in several prosomal segments (arrow). (D) Opisthosoma of an embryo injected with *CsASH2* dsRNA. HRP staining is completely lost in the opisthosoma. *l1* to *l4*, walking legs 1 to 4 (corresponding to prosomal segments 3 to 6); *o2* to *o5*, opisthosomal segments 2 to 5. Scale bar: 200 μm.

neurones in the spider is the ancestral form of neurogenesis: cells divide in the apical layer of the ventral neuroectoderm and are recruited for the neural fate at a certain point in time. An invagination site forms and eventually these cells leave the neuroectodermal layer. It can be assumed that the myriapods have a similar mode of neurogenesis, as the absence of neurone progenitor cells with the characteristics of insect neuroblasts

was also confirmed in myriapods by the analysis of mitotic activity in the ventral neuroectoderm (Whitington et al., 1991). As was mentioned above, in myriapods ventral ganglia are formed by the incorporation of so called 'ventral organs' (Dohle, 1964). In the malacostracan crustaceans, specialized neural precursor cells have evolved that are capable of dividing asymmetrically. These cells remain in the neuroectodermal layer because they also produce epidermal cells. In insects, the neuroblasts have to leave the neuroectodermal layer because these cells only produce neural cells at this point, while specialized cells, the epidermoblasts, occupy the apical position in the ventral neuroectoderm.

Proneural genes in the spider

This is the first time that proneural genes have been identified in arthropods other than insects. Similar to *Drosophila*, both identified genes are exclusively expressed in the developing nervous system. *CsASH1* transcripts can be detected in the ventral neuroectoderm before formation of the first invagination sites. A cluster of cells located in the anterior most lateral region of each hemisegment expresses the gene at this time. In this region the first five to eight invagination sites will form hours later. The medially and posteriorly extending expression domain of *CsASH1* exactly prefigures the region where the next nine to 12 invagination sites will arise. After formation of the invagination sites in that region, *CsASH1* expression is switched off and the gene is re-expressed in a lateral domain and a medial stripe in each hemisegment. Again, these expression domains prefigure the regions where the next five to eight invagination sites will form. Subsequently, expression decreases and the gene is re-expressed in a medial cluster of cells at the posterior end of each hemisegment. This expression domain covers the region that has not expressed the gene until now, but it does not cover the region where the last seven to ten invagination sites will form. Two new rows of invagination sites are added during the last wave of formation of invagination sites. However, it can be assumed that invagination sites that have formed in the medial domain are shifted to their final position owing to the compaction of the neuromeres between this stage and the end of inversion (see Fig. 9N,O,S,T). In addition, *CsASH1* has a transient expression in the invaginating cell groups. These data show that *CsASH1* is expressed in all regions of the ventral neuroectoderm where invagination sites arise. The expression pattern of *CsASH1* can therefore be compared with the expression of three genes of the ASC in *Drosophila*: *achaete*, *scute* and *lethal of scute*. These three proneural genes are expressed before delamination of the neuroblasts in partially overlapping clusters of cells. As in the spider, proneural gene expression decreases after formation of one population of neuroblasts and the genes show transient expression in the neuroblasts. Subsequently, the genes are re-expressed in regions of the ventral neuroectoderm where the next neuroblasts will form. In addition, comparable with *Drosophila*, where the proneural genes are responsible for cell shape changes in the ventral neuroectoderm before delamination of the neuroblasts (Stollewerk, 2000), the consequence of proneural gene expression in the spider seems to be a cell shape change to the bottle-like form of the invaginating cells. However, in contrast to *Drosophila*, where only one cell of a proneural cluster delaminates and becomes a neuroblast, all *CsASH1*-expressing cells enter the neural pathway.

Analysis of *CsASH1* function during neurogenesis by injection of dsRNA corresponding to this gene revealed that *CsASH1* is indeed responsible for the formation of the invagination sites. The fact that it was possible to obtain embryos that do not have any invagination sites suggests that *CsASH1* is the only gene that is involved in the recruitment of neural cells. This is in contrast to *Drosophila*, where several genes are required for the specification of the neural precursor cells.

The second proneural gene of the spider, *CsASH2*, is expressed later during neurogenesis in the invaginating cells that have already changed their morphology to a bottle-like shape. Strong expression of the gene can be detected only during a very short time window, when most of the invagination sites have already formed in the prosoma. However, the expression pattern in the opisthosoma suggests that the transcription rate of the gene is to low to be detected in younger stages with our in situ hybridisation methods. At 175 hours, 16 to 17 invagination site have formed in the first opisthosomal hemisegments. Five to eight of these invagination sites show a strong *CsASH2* expression, while the remaining invaginating cells are only weakly stained. This expression pattern suggests that the gene is expressed immediately after formation of the invagination sites but at very low levels. The expression pattern of *CsASH2* is not similar to that of the proneural genes *achaete*, *scute* and *lethal of scute* in *Drosophila*, rather it can be compared with the fourth member of the ASC, *asense*, which is not expressed until after the neuroblasts have segregated from the ventral neuroectoderm. *asense* seems to be required for the differentiation and maintenance of the specified cell fate (Alonso and Cabrera, 1988; Gonzalez et al., 1989). The observations that the neuronal HRP staining is lost and the neuropil is reduced to different degrees in embryos that have been injected with *CsASH2* dsRNA, despite the fact that the invaginating cell groups are present, suggests that *CsASH2* has a similar function in neurogenesis in the spider as *asense*, although there is only a very low degree of amino acid identity. Similarly, the vertebrate *ac-sc* homologues, *XASH1*, *MASH1* and *CASH1* are expressed relatively late in neurogenesis, being detectable only after neural progenitors have been specified (Lo et al., 1991; Ferreiro et al., 1992; Jasoni et al., 1994).

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