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Recruitment of cell groups through Delta/Notch signalling during spider neurogenesis

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SUMMARY

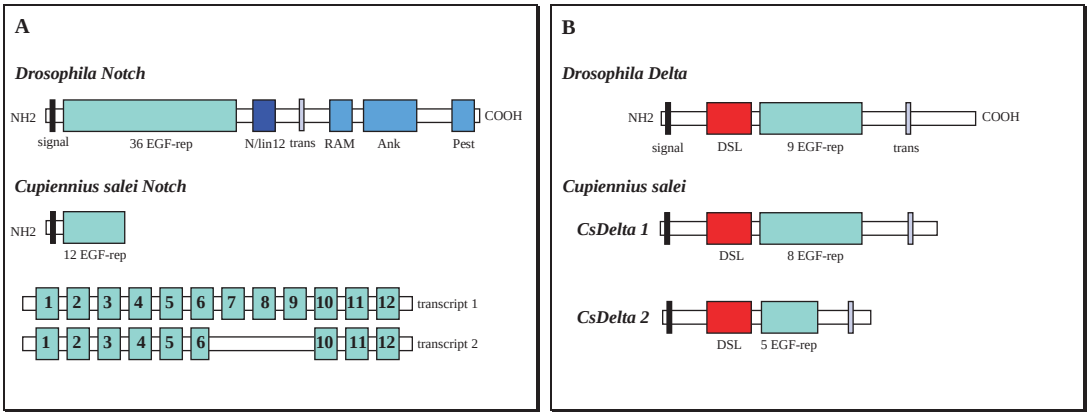
Early neurogenesis in the spider is characterised by a stereotyped pattern of sequential recruitment of neural cells from the neuroectoderm, comparable with neuroblast formation in *Drosophila*. However, in contrast to *Drosophila*, where single cells delaminate from the neuroectoderm, groups of cells adopt the neural fate and invaginate into the spider embryo. This raises the question of whether Delta/Notch signalling is involved in this process, as this system normally leads to a singling out of individual cells through lateral inhibition. I have therefore cloned homologues of *Delta* and *Notch* from the spider *Cupiennius salei* and studied their expression and function. The genes are indeed expressed during the formation of neural cells in the ventral neuroectoderm. Loss of function of either gene leads to an upregulation of the proneural genes and an altered morphology of the neuroectoderm that is comparable with *Delta* and *Notch* mutant phenotypes in *Drosophila*. Thus, although Delta/Notch signalling appears to be used in the same way as in *Drosophila*, the lateral inhibition process produces clusters of invaginating cells, rather than single cells. Intriguingly, neuroectodermal cells that are not invaginating seem to become neural cells at a later stage, while the epidermal cells are derived from lateral regions that overgrow the neuroectoderm. In this respect, the neuroectodermal region of the spider is more similar to the neural plate of vertebrates, than to the neuroectoderm of *Drosophila*.

Key words: Delta/Notch signalling, Neurogenesis, Lateral inhibition, Chelicerate, *Cupiennius salei*

INTRODUCTION

A decision between neural and epidermal fate takes place in the ventral neuroectoderm of *Drosophila*. At the beginning of neurogenesis, groups of neuroectodermal cells acquire the competence to adopt a neural fate. This process depends on the presence of proneural gene products in the neuroectodermal cells (Jimenez and Campos-Ortega, 1979; Jimenez and Campos-Ortega, 1990; Cabrera et al., 1987; Chitnis et al., 1995; Haddon et al., 1998; Chitnis and Kintner, 1996). In *Drosophila*, proneural genes are expressed in groups of four to five cells – so-called proneural clusters. One cell from each proneural cluster is singled out to become a neuroblast, while the entry into the neural pathway is blocked in the remaining cells and proneural gene expression is downregulated (Cabrera et al., 1987; Romani et al., 1987; Skeath and Carroll, 1992). Analysis of neurogenic mutants in *Drosophila* showed that the transmembrane proteins Delta and Notch restrict proneural gene expression and thus the neural fate (Marin-Bermudo et al., 1995). Binding of the ligand Delta to the Notch receptor leads to proteolytic cleavages that release the intracellular domain of Notch (Struhl and Adachi, 1998; Kidd et al., 1998; Schroeter et al., 1998; Lecourtois and Schweisguth, 1998). This intracellular domain enters the nucleus and acts as a co-activator for the transcription factor Supressor of Hairless. An important target of the activators is the *Enhancer of split* gene complex. The gene products are bHLH proteins that, when expressed in response to Notch signal transduction, inhibit the transcription of proneural genes (Ligoxygakis et al., 1998; Nakao and Campos-Ortega, 1996). As *Delta* is positively regulated by the proneural genes, Notch signalling leads to a downregulation of *Delta*. This feedback loop which links production of the ligand to activation of the receptor promotes amplification of small differences between the cells of a proneural cluster in their potential to adopt a neural fate, allowing a single cell to arise in a proneural cluster which inhibits its neighbours but is not inhibited by them (Heitzler and Simpson, 1991; Sternberg, 1993; Chitnis, 1995; Collier et al., 1996; Lewis, 1996).

This system functions in a similar manner in vertebrate neurogenesis (de la Pompa et al., 1997; Chitnis and Kintner, 1996; Chitnis et al., 1995; Haddon et al., 1998) suggesting that the singling out of individual cells may be an ancestral feature of neurogenesis that is coupled to the particular way in which Delta/Notch signalling works. However, our previous analysis of spider neurogenesis (Stollewerk et al., 2001) showed that groups of cells rather than single cells are recruited for the neural fate. This raises the question of how such group-specific recruitment of cells might be achieved and whether Delta/Notch signalling is involved in this process. Therefore, I cloned homologues of *Notch* and *Delta* to study their expression and function in spider neurogenesis.



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Drosophila  WSQDLHSSGR TDLRYSYRFV CDEHYYGEGC SVFCRPRDDA FGHFTCGERG EKMCDPGWKG QYCTDP
Xenopus     ........D. .E.K..... ...Y..... .DY..... ....S...K. ..L.N..... L...E.
CsDelta1    .T..I.KTNH .AFS.AI.VR .LDN....S. EKL....N.K ...Y..SPT. D.V.LR..T. E...IA
CsDelta2    .T..EL.TNQ ST.KLM..VY .ATN...PN. GNL......K ...Y..K.D. Q.L.K...S. S..DKA
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Fig. 1. (A,B) Comparison of the spider Delta and Notch structures to the *Drosophila* proteins and alignment of the DSL domain. (A) The *Drosophila* Notch protein contains several conserved domains. The sequence obtained so far for Notch encodes two different transcripts. One transcript lacks part of EGF repeat 6 and specifically EGF repeats 7, 8 and 9. (B) *CsDelta1* shares the highly conserved DSL domain (Delta, Serrate, Lag2) and eight EGF repeats with the fly sequence. *CsDelta2* contains the conserved DSL domain, but has only five EGF repeats. Both spider Delta proteins have a shorter intracellular domain. (C) The DSL domains of both CsDelta1 and CsDelta2 show a higher identity to the same region of the *Xenopus* X-Delta1 protein (66% and 62% identity, respectively) than to *Drosophila* (62% and 57% identity, respectively). Ank, ankyrin repeat; DSL, Delta, Serrate, Lag2 domain; EGF-rep, EGF repeats; N/lin12, Notch lin12 repeats; Pest, a region rich in proline, glutamine, serine and threonine residues; RAM, RAM23 domain.

MATERIALS AND METHODS

Cupiennius salei stocks

Fertilised females of the Central American wandering spider *Cupiennius salei Keyserling* (Cherlicerata, Arachnida, Araneae, Ctenidae) were obtained from our colony bred in Cologne. Embryos were collected as described before (Damen et al., 1998).

PCR cloning

CsDelta1, *CsDelta2* and *CsNotch* were initially found by RT-PCR on RNA prepared from 120- to 130-hour-old embryos, using degenerate primers directed against conserved positions of the DSL domain (Delta) and EGF repeats (Notch) of two invertebrate and three vertebrate neurogenic genes. We used the following primers: DL2, TWYTGYMGNCCNMGNGAYG; DL1re, CARTARTTNARR-TCYTKRTYRCA; DL2re, NWRNCCNCCCCANYYNKY; DL3re, CANGTNCCRTGNANRCANYYNGG; N7, TGYRTNTGYGTN-AAYGGNTGG; N8, GAYTGYWSNRANAAYWTHGAYG; N7re, RTTYTGRCANGGRTKNSW; N7re2, CCNKYRWANCCNGG-CATRCA. The PCR fragments were cloned and sequenced. Larger fragments of the genes were obtained by rapid amplification of cDNA ends (GeneRacer kit, Invitrogen). The sequences were deposited in the EMBL/GenBank/DDBJ databases (Accession Numbers: CsNotch, AJ507288; CsDelta1, AJ507289; CsDelta2, AJ507290).

Histology and staining

Whole-mount in situ hybridisation were performed as described (Damen and Tautz, 1999). Phalloidin staining of spider embryos was performed as has been described for flies (Stollewerk, 2000). Immunocytochemistry was performed as described (Stollewerk et al., 2001). Anti-Horseradish peroxidase antibody was purchased from Dianova (1:500) and anti-acetylated tubulin was purchased from Sigma (1:2000). Histology was performed as described (Stollewerk et al., 1996).

Double-stranded RNA interference

Preparation of double-stranded RNA, injection and further treatment of the embryo were performed as described previously (Schoppmeier and Damen, 2001; Stollewerk et al., 2001).

RESULTS

Isolation of CsNotch, CsDelta1 and CsDelta2

The conserved structural features found in Notch and Delta family members were used to amplify PCR fragments with degenerate oligonucleotide primers. The *Drosophila* Notch protein contains two conserved domains in its extracellular domain: a stretch of 36 epidermal growth factor repeats and three Lin-12/Notch repeats (Fig. 1A). With degenerate primers directed against EGF repeats 9 and 12, PCR fragments of 462 bp length were amplified. Rapid amplification of the 5′ ends of these fragments (5′RACE) yielded a 1600 bp fragment with a 1259 bp open reading frame (ORF). The obtained sequence covers the complete N-terminal region and the first twelve EGF repeats (Fig. 1A). Interestingly, the *Cupiennius Notch* (*CsNotch*) gene encodes two different transcripts. One of the transcripts lacks a part of the EGF repeat six and, specifically, the EGF repeats seven, eight and nine (Fig. 1A). The two different Notch transcripts may be the products of alternative splicing. The deduced amino acid sequence of the gene has 66% identity to the *Notch* homologue of the Australian sheep blowfly *Lucilia cuprina* (Davies et al., 1996).

The extracellular domain of the *Drosophila* Delta protein contains nine EGF repeats and the highly conserved DSL domain (Delta-Serrate-Lag2), a cystein-rich region that is required for binding to the Notch receptor (Fehon et al., 1990) and has been found in all Notch ligands identified so far (Fig. 1B). Degenerate primers directed against the DSL domain and the second EGF repeat resulted in amplification of 504 bp and 534 bp PCR fragments that fall into two groups and could be distinguished by differences at 25 amino acid positions. In order to isolate the whole sequences, I performed 5′ and 3′RACE using transcript specific primers directed against the two different PCR fragments. With this method, two Delta genes were identified in the spider: *Cupiennius salei Delta1*

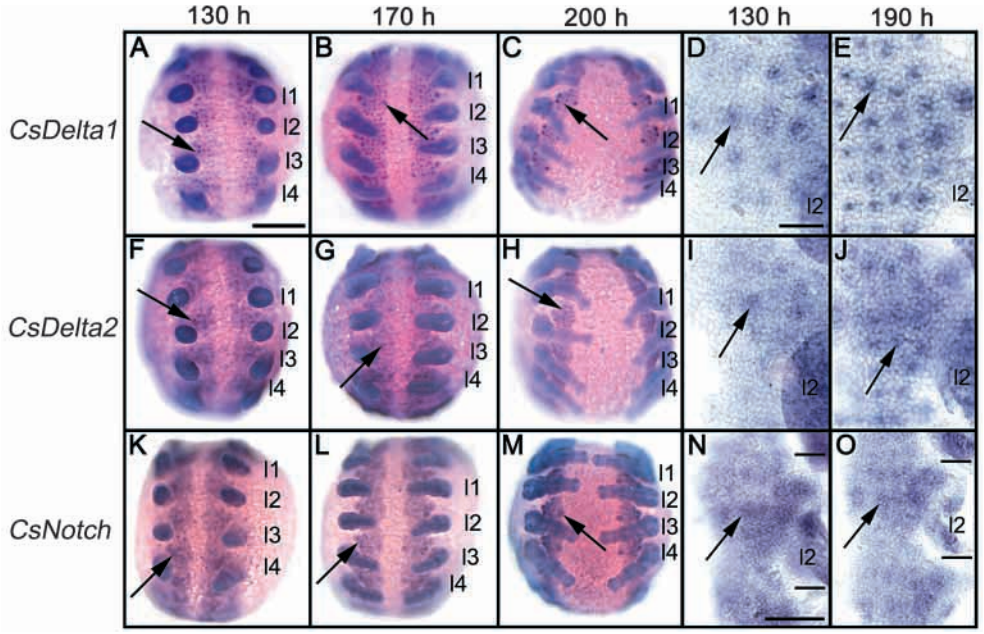

Fig. 2. (A-O) Expression pattern of the neurogenic genes in the prosoma. Whole-mount in situ hybridisation; anterior is towards the top. (A-E) *CsDelta1* expression; (F-J) *CsDelta2* expression; (K-O) *CsNotch* expression. (A,D) At 130 hours, *CsDelta1* expression is visible in the first five to eight invaginating cell groups that have formed per hemisegment (arrows). (B) Seventeen to 20 additional invagination sites (arrow) were generated at 170 hours, all of them expressing *CsDelta1*. (C) When the apical cell processes of the invaginating cells lose contact to the apical surface at about 190 hours in the prosoma, *CsDelta1* expression decreases, although some of the invaginating cell groups still show a strong expression (arrow). (E) At 180 hours, the final number of 30 to 32 invagination sites is visible. *CsDelta1* transcripts can be detected in all invaginating cell groups (arrow). (F,I) *CsDelta2* is uniformly expressed in the ventral neuroectoderm before formation of invagination sites (data not shown). At 130 hours, *CsDelta2* shows stronger expression in the invaginating cell groups (arrows), although it is still expressed in all neuroectodermal cells. (G,J) The upregulation of *CsDelta2* expression is also visible in subsequent invagination groups. (H) Expression of *CsDelta2* decreases at the same time (about 190 hours) in the invaginating cells as *CsDelta1* expression. (K,N) *CsNotch* is expressed in the whole ventral neuroectoderm at 130 hours, but shows a slightly stronger expression in the lateral regions of the hemisegments (arrows). (L,O) *CsNotch* is still expressed in all neuroectodermal cells at 170 and 180 hours, but there is heterogeneity in the expression levels (arrows). (M) The same distribution of *CsNotch* transcripts is visible at about 190 hours, but in addition a medial group of cells shows strong *CsNotch* expression (arrow). l1 to l4, walking legs 1 to 4 (corresponding to prosomal segments 3 to 6). Scale bars: in A, 200 μm for A-C,F-H,K-M; in D, 25 μm for D,E,I,J; in N, 50 μm N,O.

(*CsDelta1*) and *Delta 2* (*CsDelta2*). The 2447 bp sequence obtained for *CsDelta1* encodes a deduced protein of 683 amino acids. It shares the highly conserved DSL domain (Delta-Serrate-Lag2) and eight EGF repeats with the fly sequence (Fig. 1B). The 2115 bp *CsDelta2* sequence encodes a protein of 437 amino acids. Although it contains the conserved DSL domain in its extracellular part, the deduced protein sequence can only be aligned with the first five EGF repeats of other species, while the remaining C-terminal part is not conserved. An amino acid sequence comparison of the DSL domains of CsDelta1 and CsDelta2 with the same region in the *Drosophila* protein (Vaessin et al., 1987) indicates that CsDelta1 and CsDelta2 have 62% and 57% identity to *Drosophila* Delta, respectively. The DSL domains of both proteins show the highest identity to the same region of *Xenopus X-Delta-1* (Fig. 1C) (Chitnis et al., 1995). Besides the DSL domain, regions of greater identity to Delta proteins of other species exist between the first five EGF repeats, while the intracellular domains of both spider Delta proteins are highly dissimilar.

Expression patterns of *CsDelta1*, *CsDelta2* and *CsNotch*

In contrast to *Drosophila*, where single neuroblasts delaminate into the embryo during neurogenesis, groups of cells invaginate from the ventral neuroectoderm of the spider (Stollewerk et al., 2001). These invagination sites consist of five to nine neural precursor cells and are generated in four subsequent waves over approximately 3 days, beginning at 130 hours [stages according to Seitz (Seitz, 1966)]. The proneural gene *CsASH1* is responsible for the recruitment of the neural precursor cells and is expressed in the appropriate regions of the ventral neuroectoderm prior to the formation of invagination sites (Stollewerk et al., 2001). Expression of *CsDelta1* starts at about 130 hours after egg laying in the first five to eight groups of cells that are going to invaginate from each hemisegment (Fig. 2A,D). The transcripts can also be detected in all invaginating cell groups that are generated in the subsequent waves (Fig. 2B,C,E). The cell processes of the invaginating cells detach from the apical surface at about 200 hours in the prosoma. At that time, *CsDelta1* expression decreases, although some of the invaginating cell groups still show a strong expression (Fig. 2D). By contrast, *CsDelta2* is uniformly expressed in the ventral neuroectoderm during formation of the invagination sites, but shows a stronger expression in the invaginating neural precursors throughout neurogenesis (Fig. 2F-J). Expression of *CsDelta2* decreases at the same time (at about 200 hours) in the invaginating cells as *CsDelta1* expression, although transcripts are still visible in the neuroectodermal cells that remain apical (Fig. 2H).

As the fragment that is missing in the second *CsNotch* transcript is too small for whole-mount in situ hybridisation, the specific distribution of the two transcripts could not be resolved. Therefore, all *CsNotch*-expressing cells were localised by whole-mount in situ hybridisation using digoxigenin-labelled riboprobes corresponding to the complete 5′ region up to EGF repeat 12. Like *CsDelta2*, *CsNotch* is expressed in the whole ventral neuroectoderm at 130 hours, but shows a slightly stronger expression in the neuroectodermal

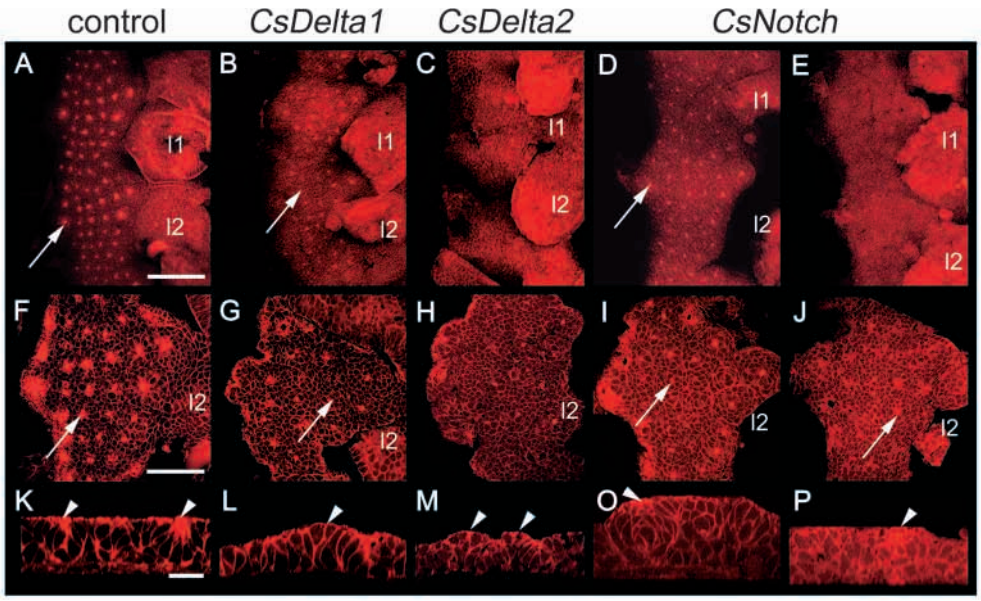

Fig. 3. (A-P) Phenotypic analysis of embryos stained with phalloidin-rhodamine after injection of *CsDelta1*, *CsDelta2* and *CsNotch* dsRNA. (A-J) Confocal micrographs of flat preparations of prosomal segments of 180-hour-old embryos; anterior is towards the top. (K-P) Transverse sections through the fourth prosomal segments; medial is towards the left. Prosomal regions of embryos injected with *GFP* dsRNA as a control (A,F,K), with *CsDelta1* dsRNA (B,G,L), *CsDelta2* dsRNA (C,H,M) and *CsNotch* dsRNA (D-P), respectively. (F-J) Higher magnifications of the fourth prosomal hemisegments. (A,F) After injection of *GFP* dsRNA, the ventral neuroectoderm shows the normal number of invagination sites (about 30 per hemisegment; dots of high phalloidin-rhodamine staining, arrows). (B,G) After injection of *CsDelta1* dsRNA, the number of invagination sites is reduced in individual segments (arrows). (C,H) A more severe reduction of invagination sites can be detected after injection of *CsDelta2* dsRNA; invagination sites are absent in the whole ventral neuroectoderm. (D,I,) After injection of *CsNotch* dsRNA, dots of high phalloidin-rhodamine staining can be detected in the positions that correspond to invagination sites in control injected embryos (arrows), although they are much smaller. (E,J) In a more severely affected embryo there is only diffuse phalloidin-rhodamine staining visible in positions that correspond to invagination sites in control injected embryos (arrow). (K) Confocal micrograph of a transverse optical section through an invagination site (arrowheads) of an embryo injected with *GFP* dsRNA. The cell processes of the basally enlarged cells extend to the apical surface. (L) Transverse optical section through the fourth prosomal hemisegment of an embryo injected with *CsDelta1* dsRNA, showing that in a region where invagination sites are missing the neuroectoermal cells form a bulge (arrowhead). (M) Transverse section through the fourth prosomal hemisegment of an embryo injected with *CsDelta2* dsRNA. Two bulges of neuroectermal cells are visible (arrowheads). (O,P) The transverse sections through the fourth prosomal hemisegments of embryos injected with *CsNotch* dsRNA reveal that, although dots of higher phalloidin-rhodamine staining are visible on the apical surface (arrowheads), there are no bottle-like cells visible underneath these dots. The presence of several cell layers suggests that cells that normally invaginate occupy space in the apical layer so that newly formed cells were pushed basally. *l1*, *l2*, walking legs 1 to 2 (corresponding to prosomal segments 3 and 4). Scale bars: in A, 150 μm for A-E; in F, 50 μm for F-J; in K, 20 μm for K-P.

regions where the first invagination sites arise (Fig. 2K,N). After formation of most of the invagination sites these domains of higher Notch expression are reduced to small regions at the lateral anterior edge of each neuromere (Fig. 2M,O). At that time *CsNotch* is still expressed in all neuroectodermal cells, although there is heterogeneity in the expression levels (Fig. 2O). The same distribution of *CsNotch* transcripts is visible at about 200 hours, but, in addition, a medial group of cells shows strong *CsNotch* expression (Fig. 2M). In summary, *CsDelta1*, *CsDelta2* and *CsNotch* are expressed during neurogenesis in a spatiotemporal pattern, indicating that they are involved in the specification of neural precursors in the spider ventral neuroectoderm.

Functional analysis of *CsDelta1*, *CsDelta2* and *CsNotch*

To analyse the function of the spider neurogenic genes, I injected double-stranded RNA (dsRNA) of *CsDelta1*, *CsDelta2* and *CsNotch*, respectively, to interfere with endogenous gene function (Fire et al., 1998; Schoppmeier and Damen, 2001). Injected embryos were cultivated until about 190 hours after egg laying (Table 1). The resulting phenotypes were analysed by staining 10% of the embryos with phalloidin-rhodamine, a dye that stains the actin cytoskeleton, and 20% with neural anti-Horseradish peroxidase antibodies (anti-HRP). The remaining 70% were hybridised with the spider proneural gene *CsASH1* (Stollewerk et al., 2001). Morphological analysis of the resulting phenotypes in the confocal laser-scanning microscope revealed that the invagination sites are missing to different degrees in embryos injected with dsRNA of the neurogenic genes (Fig. 3). Embryos injected as a control with dsRNA corresponding to a fragment of the green fluorescent protein (GFP) exhibit

Table 1. Summary of RNAi experiments

	Injected	Developed	Specific phenotype	Nonspecific phenotype	Normal
GFP	238 (100%)	179 (75%)	0 (0%)	54 (30%)	124 (70%)
CsDelta1	235 (100%)	180 (77%)	63 (35%)	57 (32%)	60 (33%)
CsDelta2	287 (100%)	191 (66%)	121 (64%)	35 (18%)	35 (18%)
CsNotch	318 (100%)	218 (68%)	106 (49%)	62 (28%)	50 (23%)

Injected, the number of embryos that were injected either with *GFP* or *CsDelta1*, *CsDelta2* and *CsNotch* dsRNA; developed, the number of embryos that developed further up to the time where they were analysed (about 190 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.

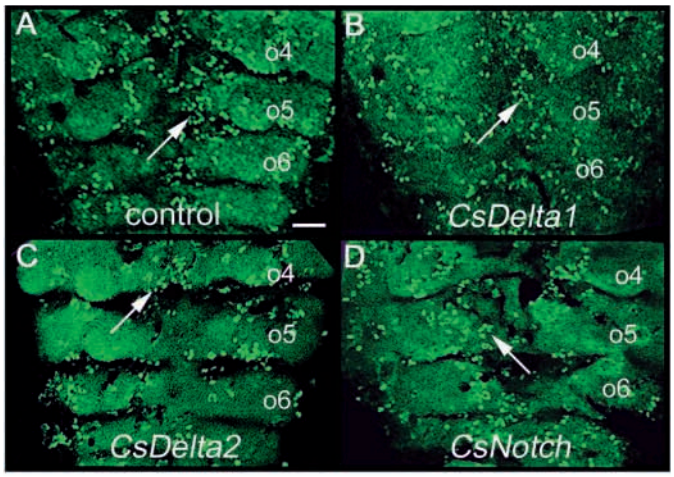

Fig. 4. (A-D) Distribution of phospho-histone 3 antigen in embryos injected with CsDelta1, CsDelta2 and CsNotch dsRNA. Confocal micrographs of flat preparations of opisthosomal segments of 160-hour embryos; anterior is towards the top. (A) The anti-phospho-histone 3 antibody stains single mitotic cells (arrow; green) that are uniformly distributed over the whole ventral neuroectoderm throughout neurogenesis in embryos injected with *GFP* dsRNA as a control. (B-D) The overall mitotic pattern is unchanged after injection of *CsDelta1*, *CsDelta2* and *CsNotch*, respectively (arrows). Scale bar: 50 μm.

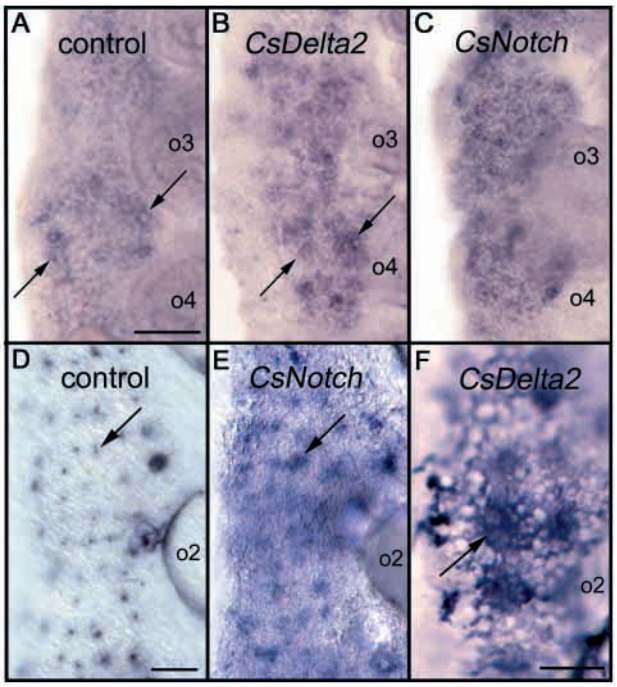

Fig. 5. (A-C) Expression pattern of the proneural gene *CsASH1* and the neural HRP antigen after injection of *CsDelta2* and *CsNotch* dsRNA. Light micrographs of flat preparations of the second to fourth opisthosomal segments; anterior is towards the top. (A) At about 180 hours, *CsASH1* is expressed in a medial stripe and a patch of cells in the lateral region of the opisthosomal hemisegments (arrows). This pattern is unchanged after injection of *GFP* dsRNA. (B,C) In embryos injected with *CsDelta2* dsRNA (B) and *CsNotch* dsRNA (C), a strong upregulation of *CsASH1* expression can be observed (arrows), while after injection of *CsDelta1* only a minor change in the expression pattern is visible (data not shown). (D) The anti-HRP antibody stains the cell processes of the invaginating cells (arrow) in an embryo injected with *GFP* dsRNA as a control. (E) After injection of *CsNotch* dsRNA, the HRP antigen is upregulated in the apical cells. A stronger staining (arrow) is visible in the regions where invagination sites normally form (compare with Fig. 3J). (F) Higher magnification of a neuroectodermal region, where no invagination sites have been formed after injection of *CsDelta2* dsRNA. Apical located cells are stained with the anti-HRP antibody (arrow). o2-o4, opisthosomal segments 2 to 4. Scale bars: in A, 100 μm for A-C; inD, 50 μm for D,E; in F, 20 μm for F.

the normal number of invagination sites in the ventral neuroectoderm, as can be seen by the dots of high phalloidin-rhodamine staining (Fig. 3A,F,K). Groups of invaginating cells that extend their cell processes to the apical surface are located underneath these dots (Stollewerk et al., 2001). The spot-like phalloidin-rhodamine staining is due to the constricted cell processes of these bottle-like shaped cells (Fig. 3K).

In 35% of the embryos that were injected with *CsDelta1* dsRNA, the number of invaginating cell groups is reduced in individual segments (Fig. 3B,C). A more severe reduction of invagination sites can be detected after injection of *CsDelta2* dsRNA: in 47% of all embryos that show a specific phenotype, invagination sites are absent in the whole ventral neuroectoderm (Fig. 3C). Transverse sections show that the morphology of the ventral neuroectoderm of embryos injected with dsRNA of any neurogenic gene is altered in the same way as in *Drosophila* neurogenic mutants (Stollewerk, 2000): the neuroectoderm forms bulges (Fig. 3L,M) because the cells that normally invaginate occupy a space in the apical layer. As the proliferation rate is not affected in these embryos (Fig. 4), newly formed cells are either pushed to the apical (Fig. 3L,M) or to the basal side (Fig. 3O,P).

After injection of *CsNotch* dsRNA, dots of high phalloidin-rhodamine staining can be detected in the positions that correspond to invagination sites in control embryos, although they are much smaller (Fig. 3D,I). In more severely affected embryos (Fig. 3E) there is only a diffuse phalloidin-rhodamine staining visible in positions where invagination sites form in control injected embryos (Fig. 3J). However, transverse optical sections through the neuroectoderm of embryos injected with *CsNotch* dsRNA revealed that invaginating cells with bottle-like shapes are missing (Fig. 3O,P). This indicates that groups of cells attach to each other at the apical surface, but the process of invagination is disturbed after injection of *CsNotch* dsRNA.

Alterations in the distribution of proneural gene transcripts and HRP antigen after loss of neurogenic gene function

In *Drosophila* neurogenic mutants the transcriptional repression of proneural genes to single cells of the proneural clusters fails to occur resulting in an overproduction of neuroblasts at the expense of epidermal cells (Marin-Bermudo et al., 1995; Brand and Campos-Ortega, 1988). Similar to *Drosophila*, the spider proneural gene *CsASH1* is expressed in patches of cells before each wave of formation of invagination sites. The expression becomes restricted to the invaginating cells, before the gene is re-expressed in regions where the next invagination sites will form. I analysed

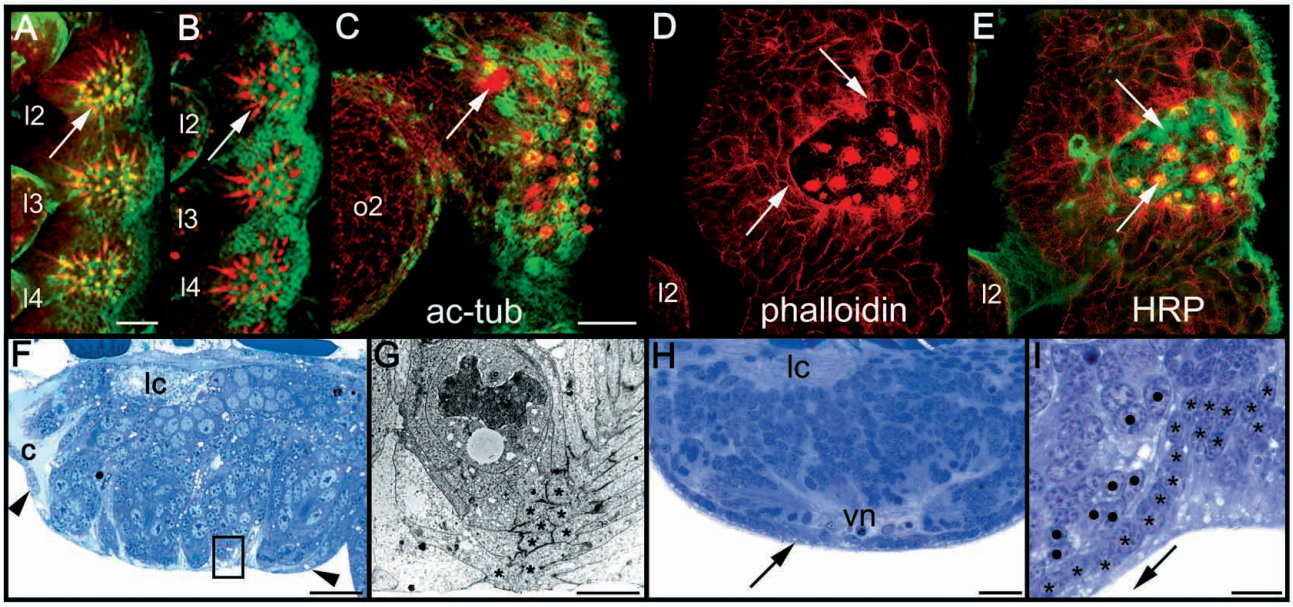

Fig. 6. (A-E) Expression patterns of the neural anti-HRP and anti-acetylated tubulin antibodies (green) and morphology of the neuromeres; double-staining with phalloidin-rhodamine (red). Confocal micrographs of flat preparations of prosomal neuromeres (A,B,D,E), an opisthosomal neuromere (C) and light (F,H,I) and electron micrographs (G) of transverse sections through the fourth prosomal neuromere at 240 hours (A-C), 280 hours (D,E), 250 hours (F,G,I) and 300 hours (H); anterior is towards the top (A-E); medial is towards the left (F-I). (A,E) The HRP antigen is expressed on the axons of the invaginated cells and in addition in the apical cells (arrows). (B,C) Acetylated tubulin is expressed in the cells that remain in the outer layer, but not in the axons of the invaginated cells (arrows). (D,F) The epidermis overgrows the neuromeres at the end of neurogenesis (arrows in D; arrowheads in F). (G) Higher magnification of a similar region framed in F. Ultrastructural analysis reveals that the neurones and axons (asterisks) are not covered with epidermal cells. (H) At 300 hours the epidermal cells have completely overgrown the ventral neuromers. (I) At 250 hours the epidermoblasts (asterisks) can be distinguished morphologically from the surrounding cells. Neurones at the border of the ventral neuromer are marked with black dots. l2, walking leg 2 corresponding to the fourth prosomal segment; o2, second opisthosomal segment; lc, longitudinal connective; c, commissure; vn, ventral neuropil. Scale bars: in A, 100 μm for A,B; in C, 50 μm for C-E; in F, 20 μm for F; in G, 4 μm for G; in H, 20 μm for H; in I, 15 μm for I.

the expression pattern of *CsASH1* in embryos injected with dsRNA of the neurogenic genes to see whether the restriction of *CsASH1* expression to the invaginating neural precursors is a function of the spider neurogenic genes. At about 180 hours, *CsASH1* is expressed in a medial stripe and a patch of cells in the lateral region of the opisthosomal hemisegments. This pattern is unchanged after injection of GFP dsRNA (Fig. 5A). In embryos injected with *CsDelta2* and *CsNotch* dsRNA, respectively, a strong upregulation of *CsASH1* expression can be observed (Fig. 5B,C), while after injection of *CsDelta1* only a minor change in the expression pattern is visible (data not shown).

To determine whether the neuroectodermal cells differentiate into neurones after interference with neurogenic gene function, injected embryos were stained with neural anti-HRP antibodies. The expression of the HRP antigen is restricted to the invaginating cells during the first phase of neurogenesis (see below). A spot-like HRP staining is visible in the apical region of the ventral neuroectoderm that corresponds to the constricted cell processes of the invaginating neural precursor cells. This pattern is unchanged after injection of *GFP* dsRNA (Fig. 5D). After injection of dsRNA of the neurogenic genes, there is an excess of anti-HRP staining in the apical cell layer (Fig. 5E,F), although *CsDelta1* injected embryos show minor alterations in the staining pattern (data not shown). In addition, in embryos injected with *CsNotch* dsRNA a stronger staining is visible in the regions where invagination sites normally form (Fig. 5E).

In summary, functional analysis shows that Delta/Notch signalling mediates lateral inhibition in the ventral neuroectoderm of the spider in the same manner as in *Drosophila*.

No decision between epidermal and neural fate in the spider ventral neuroectoderm

In *Drosophila*, the neuroectodermal cells have a choice to develop as neuroblasts or as epidermoblasts (Campos-Ortega, 1993). The cells that are not singled out for the neural fate, remain in the outer layer and differentiate into epidermal cells. To analyse the fate of the neuroectodermal cells that do not invaginate into the spider embryo, I investigated the morphology of the ventral neuroectoderm after the process of invagination is completed. Light and electron microscopic analyses (Fig. 6F,G) revealed that the ventral nerve cord was not covered with epidermis at 220 hours, when the invaginated cells have already formed a neuropil. Instead, the epidermal cells arise lateral and medial to the ventral neuroectoderm and overgrow the neuromeres between 250 and 300 hours (Fig. 6D,F,H,I). Medial to the ventral neuroectoderm, epidermal cells that have covered the space between the separated halves of the germband are shifted over the neuromers as the split germbands move towards each other on the ventral surface during late embryogenesis (Fig. 6F, arrowhead). Lateral to the

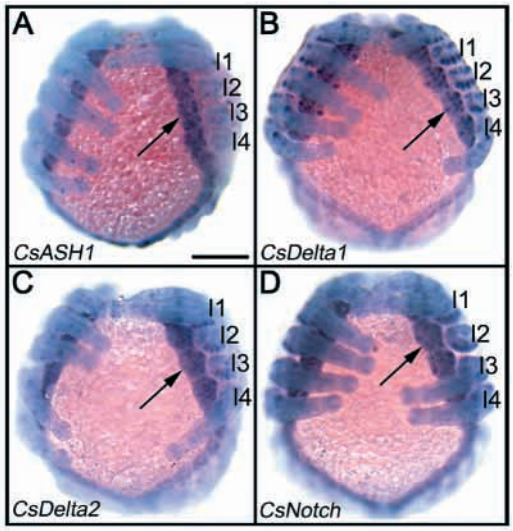

Fig. 7. (A-D) Expression pattern of the proneural gene *CsASH1* and the neurogenic genes during the second phase of neurogenesis. Whole-mount in situ hybridisation; anterior is towards the top. (A) The proneural gene *CsASH1* is re-expressed in the apical neuroectderm after the process of invagination is completed. (B) *CsDelta1* is still expressed in a subset of the invaginated cells. Transcripts are visible in about 7 axon fascicles per hemisegement (arrow). (C,D) *CsDelta2* (C) and *CsNotch* (D) are uniformly expressed in the neuroectoderm (arrows). l1 to l4, walking legs 1 to 4. Scale bar: 200 μm.

neuromers, epidermal cells are generated that start to spread medially over the neuromers at 250 hours (Fig. 6I, asterisks). The cells that do not invaginate but remain in the apical cell layer re-express the proneural gene *CsASH1* at about 220 hours (Fig. 7A). In addition, *CsDelta2* and *CsNotch* are expressed in all cells of the outer layer (Fig. 7C,D), while *CsDelta1* is still expressed in a subset of the invaginated cells: transcripts are visible in about seven axon fascicles per hemisegment (Fig. 7B). About 20 hours later, the cells remaining apical differentiate into neural cells as can be shown by staining embryos with the neural marker antibodies anti-HRP and anti-acetylated tubulin (Fig. 6A,B). Interestingly, these antigens are differentially expressed. While the HRP antigen is expressed in the axons of invaginated cells and in the cells remaining apical (Fig. 6A,E), acetylated tubulin can only be detected in the apical cells (Fig. 6B,C).

These results suggest that there is no decision between an epidermal and a neural fate in the ventral neuroectoderm of the spider, as the epidermis does not arise until neurogenesis is completed. The cells that remain in the apical layer enter a second phase of neurogenesis expressing the genes that are involved in singling out neural precursors.

DISCUSSION

Identification of *Cupiennius salei Notch* and *Delta*

This is the first time that *Notch* and *Delta* genes have been identified in arthropods other than insects. The deduced amino acid sequence of the *CsNotch* fragment obtained so far shows significant similarity to the same region of the two known insect *Notch* proteins from *Drosophila melanogaster* and *Lucilia cuprina*. The Notch protein contains several conserved domains whose functions have been studied both genetically and biochemically. The extracellular domain consists of a stretch of 36 EGF-like repeats and three lin-12/Notch repeats (Kidd et al., 1986; Wharton et al., 1985). The maintenance of individual forms of particular EGF repeats during evolution of the Notch protein seems to be important for its function, as corresponding EGF repeats in Notch proteins of different species show more similarity to each other than to adjacent repeats in the same protein (Lardelli et al., 1994). By genetic analysis, different functions have been assigned to individual EGF repeats. A region centred around EGF repeats 10 to 12 has been shown to be essential and sufficient for binding to the ligand Delta and thus is important for the function of Notch in lateral inhibition (Rebay et al., 1991; Brennan et al., 1997). A second region consisting of EGF repeats 24 to 26 has been associated with another function of Notch: the induction of proneural clusters (Brennan et al., 1997). The finding that two Notch transcripts are present in the spider, one of which lacks three EGF repeats, is interesting in view of the structure of the vertebrate Notch genes. In the mammalian Notch 4 protein, for example, EGF repeats equivalent to EGF repeats 14 to 27 of the *Drosophila* molecule have been replaced by eight EGF repeats that are unique to Notch 4, thus removing the functional domain involved in induction of proneural clusters (Uyttendaele et al., 1996). The mouse Notch 3 protein lacks EGF repeat 21 and an EGF repeat-sized region covering parts of EGF repeats 2 and 3 (Lardelli et al., 1994). As *Drosophila* mutants that map to these EGF repeats are missing, the functional significance of the deletions is unknown. Although there are also no *Drosophila* mutations known that affect the EGF repeats 7, 8 and 9 missing in the smaller *Notch* transcript of the spider, it can be assumed that the ligand-binding specificity of these transcript differs from that of the larger transcript, because it has been shown that individual EGF repeats are important for protein-protein interactions in other systems (Mayer et al., 1993).

The two identified spider *Delta* homologues both contain the highly conserved DSL domain that is required for binding of Delta to Notch (Fehon et al., 1990). In common with the vertebrate Delta proteins, CsDelta1 only has eight EGF repeats as compared with nine repeats in *Drosophila*, while CsDelta2, in addition, lacks three EGF repeats and is significantly divergent from *Drosophila Delta* and its other homologues.

Expression patterns of neurogenic genes correlate with formation of neural precursors

During neural cell fate specification in *Drosophila*, Notch and its ligand Delta appear to be evenly expressed in proneural regions, reflected in both RNA and protein distributions (Baker, 2000). Although it has been proposed that within a proneural cluster the cell expressing the highest amount of *Delta* is selected for the neural fate, no modulation in Delta expression has yet been observed in the ventral neuroectoderm of fly embryos. By contrast, the expression patterns of the zebrafish *delta* genes can be correlated to the formation of neural precursors. While *deltaB* is expressed strongly and selectively in the neural precursors, *deltaA* is expressed more diffusely, in patches of cells showing a heterogeneous expression level (Haddon et al., 1998). The cells within a *deltaA* patch that

expresses *deltaA* strongly are precisely the nascent neurones that, in addition, express *deltaB*. A similar expression pattern of *Delta* genes is visible in the ventral neuroectoderm of the spider. While *CsDelta1*, like zebrafish *deltaB*, is exclusively expressed in neural precursors, *CsDelta2* transcripts are distributed uniformly throughout the neuroectoderm and accumulate in nascent neurones similar to zebrafish *deltaA*. However, there is one major difference: whereas high amounts of *deltaA* and *deltaB* transcripts can only be detected in scattered cells in the zebrafish, groups of cells express high levels of *CsDelta1* and *CsDelta2* in the spider ventral neuroectoderm. *CsNotch* is expressed in all neuroectodermal cells, but shows stronger expression in the regions where the first invagination sites form. This expression resolves into a more uniform distribution of transcripts during the subsequent waves of invagination.

A similar mechanism with different outcome

Loss-of-function experiments show that similar to *Drosophila* and the vertebrates the neurogenic genes of the spider regulate neurogenesis through a mechanism of lateral inhibition limiting the proportion of cells that segregate at each wave of neural precursor formation. However, in contrast to *Drosophila* and also to vertebrates, where Delta/Notch signalling has only been shown to specify differences in single cells within a field of initially equivalent cells, in the spider groups of cells adopt the neural fate simultaneously (Fig. 8).

According to current models, lateral inhibition is thought to operate competitively because of a feedback loop that amplifies any initial differences between neighbouring cells: a cell expressing more Delta activates Notch more strongly in its neighbours. Notch activation in these cells inhibits not only their differentiation, but also expression of Delta, thereby reducing their ability to deliver lateral inhibition (Chitnis, 1995; Heitzler and Simpson, 1991; Sternberg, 1993; Ghysen et al., 1993). The higher expression of *CsDelta2* and exclusive expression of *CsDelta1* in a group of adjacent cells suggests that Notch signalling must be inactive in the invaginating cell groups of the spider, as these cells would otherwise inhibit each other from adopting a neural fate. The inhibitory effect of Delta was demonstrated by ectopically expressing *X-Delta-1* in the neural plate of *Xenopus* embryos leading to a suppression of primary neurogenesis (Chitnis et al., 1995).

It has been suggested, however, that the Delta to Notch ratio within a cell determines its ability to receive Notch signalling. Doherty and co-workers (Doherty et al., 1996) observed that in the *Drosophila* wing imaginal disc Notch signalling is strongest between cells that express high levels of Delta and cells with low levels of Delta. The authors suggest that signalling only occurs when cells with a Delta/Notch ratio low enough to allow signal reception are juxtaposed to cells expressing high levels of Delta. In addition, Heitzler and Simpson (Heitzler and Simpson, 1991) showed that in mosaic animals Notch mutant cells have a stronger capacity to send inhibitory signals than their wild-type neighbours. These data support a model in which high proneural gene expression in groups of neuroectodermal cells leads to an enhancement of *CsDelta2* expression and a simultaneous activation of *CsDelta1* expression in the spider. This, in turn, results in a shift in the Delta/Notch ratio within these cells, making them insensitive to Notch signalling.

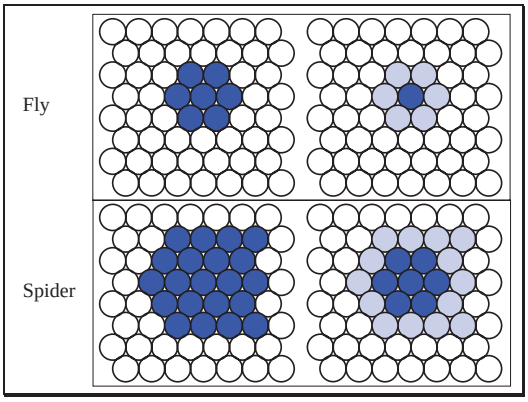

Fig. 8. Comparison of recruitment of neural precursors in fly and spider. (Top) In *Drosophila*, Delta/Notch signalling specifies differences in a single cell within a field of initially equivalent cells leading to the recruitment of one neural precursor (neuroblast) from a proneural cluster. (Bottom) In the spider, groups of cells that are located within a field of initially equivalent cells adopt the neural fate simultaneously. Dark blue, cells that have the competence to adopt the neural fate; light blue, cells that have not adopted the neural fate.

Despite the fact that in the spider Delta/Notch signalling leads to groups of five to nine cells adopting the neural fate, when compared with the selection of single neuroblasts in the fly, there is only a minor difference in the overall number of neurones generated per ventral hemisegment in *Drosophila* and *Cupiennius*. The reason is that the approximately 30 insect neuroblasts delaminate into the embryo and divide several times to give rise to about 200 neurones per hemisegment. By contrast, in the spider, most of the neural precursors do not divide after their invagination. As 30 to 32 invaginating cell groups per hemisegment are formed in the spider, it can be estimated that a neuromer consists of about 220 neurones on average. Comparison of neurogenesis in different insects (Thomas et al., 1984) and crustaceans (Whitington and Bacon, 1997) has revealed that a given segmental neuroblast appears to produce similar neurones even in widely divergent species. Therefore, the stereotyped positions of the neuroblasts and the invariant identity of their progeny seems to be an ancient feature that has changed little at least through the evolution of insects and higher crustaceans. However, the structure and development of the myriapod CNS shows little in common with the insect and crustacean ventral nerve cord (Whitington and Bacon, 1997). Future analysis will show whether the probably not clonally related invaginating cell groups of the spider have invariant cell fates and whether segmental neurones can be homologised to nerve cells at similar positions in insects and crustaceans.

The ventral neuroectoderm of the spider is comparable to the neural plate of vertebrates

In the ventral neuroectoderm of *Drosophila* Delta/Notch signalling is used for a decision between two cell fates: delaminating cells become neural precursors, while cells that remain apical give rise to epidermis. This decision does not take place in the spider neuroectoderm, rather, the cells remaining apical enter a second phase of neurogenesis, re-expressing proneural and neurogenic genes. Interestingly, the

early and late populations of neurones differ in their expression of neuronal antigens. Although both populations express the HRP antigen, acetylated tubulin can only be detected in neurones that are generated during the second phase of neurogenesis. This differential distribution of neural markers in primary and secondary neurones is also visible in vertebrates (Bang and Goulding, 1996).

As all cells of the neurogenic region develop into neurones, the ventral neuroectoderm of the spider can be compared with the neural plate of vertebrates. Similar to vertebrates, most cell divisions occur apical, while the neural precursors exit the cell cycle and differentiate in deeper layers (Stollewerk et al., 2001). During primary neurulation in vertebrates, the original ectoderm is divided into three sets of cells: (1) the internally positioned neural tube, which will form the brain and the spinal cord; (2) the externally positioned epidermis of the skin; and (3) the neural crest cells. A division of the ectoderm into at least two populations is also visible in the spider: the ventral neuroectodermal cells and epidermal precursors located lateral and medial to the neurogenic region.

In summary, the data show that neurogenesis in the basal arthropod *Cupiennius salei* shares features with both *Drosophila* and vertebrates. Similar to the generation of neuroblasts in *Drosophila*, invagination sites arise sequentially and in stereotyped positions in regions that are prefigured by the proneural gene *CsASH1* (Stollewerk et al., 2001). However, comparable with the neuroepithelial cells of the vertebrate neural plate, all cells of the neurogenic region of the spider seem to enter the neural pathway, while the neurogenic genes restrict the proportion of cells that adopt the neural fate at each wave of neural precursor formation.

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