Developmental Biology 371 (2012) 302–311

Contents lists available at SciVerse ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Evolution of Developmental Control Mechanisms

Unravelling the evolution of neural stem cells in arthropods: Notch signalling in neural stem cell development in the crustacean *Daphnia magna*

Petra Ungerer, Bo Joakim Eriksson [1], Angelika Stollewerk *

School of Biological and Chemical Sciences, Queen Mary, University of London, Mile End Road, London E1 4NS, UK

ARTICLE INFO

Article history:
Received 20 May 2012
Received in revised form
14 August 2012
Accepted 23 August 2012
Available online 1 September 2012

Keywords:
Notch signalling
Neurogenesis
Neuroblast differentiation
Co-option
Tetraconata

ABSTRACT

The genetic regulatory networks controlling major developmental processes seem to be conserved in bilaterians regardless of an independent or a common origin of the structures. This has been explained by the employment of a genetic toolkit that was repeatedly used during bilaterian evolution to build the various forms and body plans. However, it is not clear how genetic networks were incorporated into the formation of novel structures and how homologous genes can regulate the disparate morphological processes. Here we address this question by analysing the role of Notch signalling, which is part of the bilaterian toolkit, in neural stem cell evolution in arthropods. Within arthropods neural stem cells have evolved in the last common ancestor of insects and crustaceans (Tetraconata). We analyse here for the first time the role of Notch signalling in a crustacean, the branchiopod *Daphnia magna*, and show that it is required in neural stem cells for regulating the time of neural precursor production and for binary cell fate decisions in the ventral neuroectoderm. The function of Notch signalling has diverged in the ventral neuroectoderm of insects and crustaceans accompanied by changes in the morphogenetic processes. In the crustacean, Notch controlled mechanisms of neuroblast regulation have evolved that are surprisingly similar to vertebrates and thus present a remarkable case of parallel evolution. These new data on a representative of crustaceans complete the arthropod data set on Notch signalling in the nervous system and allow for reconstructing how the Notch signalling pathway has been co-opted from pre-existing structures to the development of the evolving neural stem cells in the Tetraconata ancestor.

Introduction

The phylogenetic relationships of arthropods have been intensely discussed for decades but recent large-scale molecular and morphological analyses seem to have settled the debate (e.g., Giribet and Egdecombe, 2011; Regier et al., 2010). It is now generally accepted that insects and crustaceans are sister groups, named Tetraconata or Pancrustacea. Myriapods are closely related to the Tetraconata and together these two groups form the Mandibulata (Suppl. Fig. 1). Based on these evolutionary relationships, we have recently analysed the evolution of neural precursors and their selection in arthropods. One of the main findings of the study was that the nervous system of arthropods is generated by different types of progenitors (Eriksson and Stollewerk, 2010). The neuroblasts of insects and crustaceans can be considered as stem cells based on the definition that stem cells divide to produce another stem cell and a committed cell which forms a restricted set of differentiated cell types (e.g., Bate, 1976; Dohle, 1976; Gilbert, 2006; Goodman and Doe, 1993; Landgraf et al., 1997; Schmidt et al., 1997; Ungerer and Scholtz, 2008). In contrast the neural progenitors of the remaining arthropods either generate two committed cells (onychophorans) or directly differentiate into neurons or glia (chelierates and myriapods) (e.g., Dove and Stollewerk, 2003; Eriksson and Stollewerk, 2010; Mayer and Whitington, 2009; Stollewerk et al., 2001). Outgroup analyses in a sister group to the euarthropods (Onychophora) and additional data from various regions of neurogenesis in euarthropods suggest that the generation of the nervous system by neural precursors lacking stem cell characteristics represents the ancestral pattern of arthropod neurogenesis (Eriksson and Stollewerk, 2010). Neural stem cells must therefore have evolved in the last common ancestor of Tetraconata (Suppl. Fig. 1).

These data suggest that neural stem cells have evolved independently within arthropods and in the lineage leading towards vertebrates. This provides a unique opportunity to understand how conserved neural networks have been co-opted to neural stem cell development and how the morphogenetic processes have

* Corresponding author.
E-mail addresses: p.ungerer@qmul.ac.uk (P. Ungerer), joakim.eriksson@univie.ac.at (B.J. Eriksson), a.stollewerk@qmul.ac.uk (A. Stollewerk).
[1] Current address: Department of Neurobiology, Faculty of Life Sciences, University of Vienna, Althanstrasse 14, 1090 Vienna, Austria.

0012-1606/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ydbio.2012.08.025

evolved that resulted in the emergence of neural stem cells. Here we focus on the role of the Notch signalling pathway in the development and regulation of neural stem cells (neuroblasts) and discuss how this pathway has been co-opted to the morphogenetic processes that generate neuroblasts in Tetraconata. While the function of Notch signalling has been analysed in detail in the developing nervous system of insects, in particular in *Drosophila* (eg., Egger et al., 2010; Hartenstein et al., 1992; Heitzler and Simpson, 1991), there are no data available on crustaceans. We therefore analyse here the role of Notch signalling in crustacean neurogenesis.

Recently, we have published the first study of neural gene expression in early neurogenesis in the branchiopod crustacean *Daphnia magna* (Ungerer et al., 2011). We discovered significant differences in the spatial and temporal expression that correlate with differences in the morphological processes of neuroblast formation. Both in insects and crustaceans neuroblasts generated in the ventral neuroectoderm divide asymmetrically to produce neural precursors (ganglion mother cells (GMCs)) which divide once to generate neurons and glial cells (Bossing et al., 1996; Doe and Goodman, 1985a,1985b; Dohle, 1976; Goodman and Doe, 1993; Schmidt et al., 1997; Scholtz, 1992; Ungerer et al., 2011; Ungerer and Scholtz, 2008). However, in contrast to insects, crustacean neuroblasts do not delaminate to form an internal layer but remain in the outer neuroepithelium and are located next to each other (Ungerer et al., 2011). These morphological differences are reflected in neural gene expression. In *Drosophila*, the *achaete-scute* genes are essential for neuroblast formation and are initially expressed in small clusters of cells (proneural clusters) in the ventral neuroectoderm (VNE) but the expression becomes restricted to single neuroblasts (Campos-Ortega, 1993). Activation of Notch signalling leads to the down-regulation of proneural genes in the cells that are not selected for the neural fate (Heitzler and Simpson, 1991). Neuroblasts delaminate and express genes required for neural differentiation (e.g. *asense*) and asymmetric division (e.g. *snail*, *prospero*) (Campos-Ortega, 1993; Southall and Brand, 2009). In contrast, in the crustacean *D. magna* the *Achaete-Scute-homologue* (*ASH*) is not expressed in proneural clusters, rather, it is up-regulated in individual neuroblasts (Ungerer et al., 2011). In addition, *Dam snail* (*sna*) is expressed first while *Dam ASH* is only expressed *after* formation of the neuroblasts indicating that it is not involved in neural cell fate determination (Ungerer et al., 2011).

Here we analyse the role of Notch signalling in the formation and regulation of neuroblasts in the ventral neuroectoderm of the crustacean *D. magna* in order to understand how the diverged morphogenetic processes are regulated in insects and crustaceans and to shed light on the ancestral pattern of Tetraconata neural stem cell regulation and thus the origin of arthropod neural stem cells.

Materials and methods

Cloning and staining

Dam N, *Dam Dl* and *Dam Hes1* to *3* were amplified by PCR on cDNA synthesised from RNA extracted from embryos. *D. magna* sequences were kindly provided by J.-C. Walser (Dieter Ebert laboratory). The Genbank accession numbers are: HQ259915, *Dam Dl*; HQ398106, *Dam N*; JN679203, *Dam Hes1*; JN679204, *Dam Hes2*; JN679205, *Dam Hes3*. In situ hybridisation, immunohistochemistry (rabbit-anti-Phospho-Histone 3; Sigma, 1:250), Hoechst/Phalloidin staining were performed as described (Ungerer et al., 2011). Confocal microscope images were acquired with a Leica SP5 (lasers: HeNe633, DPSS 561, Argon, UV) and Leica Application Suite 2.6.0 software using a HCX PLAPO lambda blue 1.4 oil objective. Image stacks were acquired at 200 Hz bidirectional sequential scan, 1024×1024 pixels with a pixel size of $240.50 \times 240.50\ nm^2$ and an optical thickness of 0.8 µm. Confocal image stacks were processed with IMARIS (Bitplane). Transmitted light and epifluorescence micrographs were taken on a Leica DM IL HC with the following objectives: PL FL $10\times$/0.30 -/D 11.0, HCX PL FL L $20\times$/0.40 CORR 0-2/C 6.9 and HCX PL FL L 40x/0.60 CORR 0–2/C. Hoechst was viewed with a UV AS Leica filter system, Cy3 with a Leica green N2.1S filter system. A Leica DFC420C 5MPixel Colour Camera and LAS 2.8.1 software were used to acquire images with 16bit colour depth, 2592×1944 pixel and JPEG format.

DAPT treatment

D. magna embryos were collected before appearance of antenna 2. *D. magna* culturing and egg collection were done as described before (Ungerer et al., 2011). Embryos were incubated in 0.75 mM DAPT (2,5-bis[4-dimethylaminophenyl]-1,3,4-thiadiazole, Sigma) in mineral water for 4 h at 25 °C. As the DAPT stock solution was dissolved in DMSO, as a control half of the embryos were incubated in an equal volume of DMSO in mineral water. Subsequently embryos were transferred to *Daphnia* medium, and development stopped at the desired stages by fixation.

Results

The sequence of neuroblast formation and generation of a preliminary neuroblast map

In order to analyse the function of Notch signalling in the development of neuroblasts in *D. magna*, we first established the position of the neuroblasts as well as the sequence of their formation in the thoracic segments. We used *Dam sna*, *Dam asense (ase)* and *Dam prospero* (*pros*) as neuroblast markers and Hoechst as a marker for all nuclei (Figs. 1–2; Suppl. Fig. 2) (Ungerer et al., 2011). By analysing the expression patterns of these genes in neurogenesis stages 2 to 4 (NS2 to NS4) (stages after Ungerer et al., 2011), we generated a preliminary neuroblast map of thoracic hemi-neuromeres. According to insect neuroblast maps, we assigned row and column numbers to each neuroblast (Fig. 1Q). However, this does not imply homology of insect and *D. magna* neuroblasts that have been assigned the same numbers. The analysis confirmed our previous data that *Dam sna* is the first neural gene to be expressed in the neuroblasts (Ungerer et al., 2011). The transition to asymmetric division is marked by the activation of *Dam pros* (Ungerer et al., 2011) (Suppl. Fig. 3). Taking into account all thoracic hemi-neuromeres, *Dam sna* expression can be detected in all neuroblasts except for NBs 2-2, 4-1, 4-2 and 7-1 in NS2 (Fig. 1A–H). At this time *Dam ase* is expressed in two neuroblasts per hemi-neuromere (NBs 5-1 and 6-1; Fig. 2A–D), while *pros* is only expressed in one neuroblast (NB 1-1; Fig. 1B, F, H; Suppl. Table 1). There is no regular sequence of neuroblast formation which is reflected in the highly variable number and order of neuroblasts expressing *Dam sna* in the thoracic hemi-neuromeres (Fig. 1). However, generally a single-cell-wide ring of neuroblasts appears first in each hemi-neuromere followed by additional neuroblasts that fill the gaps between existing neuroblasts (Fig. 1D, H, L, Q). In addition, neuroblasts arise medially and/or laterally to existing neuroblasts so that the ring-like expression domain expands to two cells in width by the end of NS2 (Fig. 1L, P, Q). Some neuroblasts (*Dam sna+*) divide symmetrically in the plane of the VNE (Fig. 3A, B).

At NS3, *Dam sna* is expressed in all neuroblasts except for NB 7-1, again taking into account all thoracic hemi-neuromeres (Fig. 1I–L; Suppl. Fig. 2A, B). The number of *Dam ase* expressing

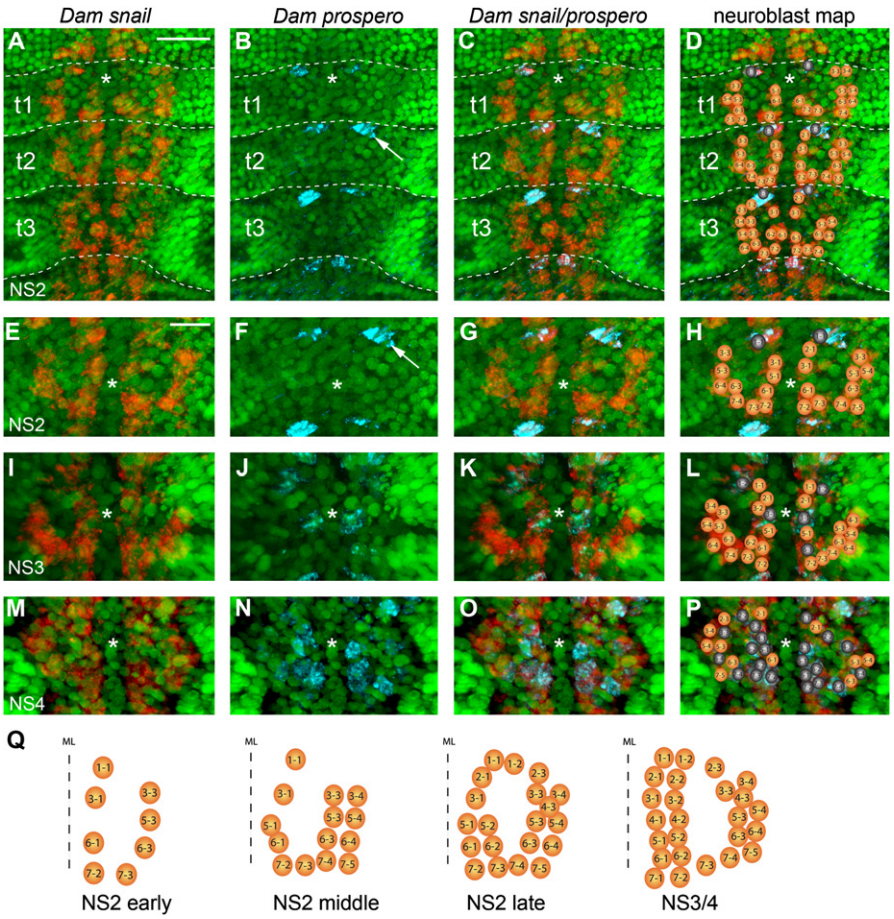

Fig. 1. (A–Q) *Daphnia magna* formation and arrangement of neuroblasts in thoracic neuromeres. (A–P) Confocal micrographs of *D. magna* embryos stained with DIG/fluorescein-labelled RNA probes of *Dam sna* (red) and *Dam pros* (blue), Hoechst (green) for nuclei; anterior is towards the top. Asterisks indicate the ventral midline. Dashed horizontal lines indicate the segmental borders in A–D. Magnifications of individual thoracic segments are shown in E–P. Overviews of thoracic segments of the embryos shown in I–P are presented in Suppl. Fig. 2. Column 1 and 2 show the single channels, column 3 the overlay and column 4 the neuroblast labelling (orange spheres: *Dam sna*+ neuroblasts; grey spheres: *Dam sna*+ and *Dam pros*+ in D, H, L, P). During neuroblast formation the thoracic hemi-neuromeres consist of 7 rows of about 5 to 6 cells each. Since the neuroblasts are formed in the VNE and do not delaminate, they can be assigned to the corresponding rows. (A–D) Same embryo, stage NS2, showing an overview of the arrangement of neuroblasts in 3 thoracic segments. The arrow in B points to the *Dam pros*+ neuroblast NB 1-1. (E–H) Same embryo as in A–D. Magnification of the second thoracic segment. The arrow in F points to the *Dam pros*+ neuroblast NB 1-1. Please note that this neuroblast has a large cell body which extends along the medio-lateral axis. (I–L) The images show the 4th thoracic segment of an embryo at late NS3. Additional neuroblasts express *Dam pros*. (M–P) 3rd thoracic segment of an embryo at stage NS4. (Q) Arrangement of neuroblasts (orange spheres) and sequence of formation. *t1 to t3*, thoracic segments 1 to 3. Scale bars: (A) 50 μm in A–D; (E) 25 μm in E–P. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

neuroblasts is considerably increased compared to NS2. *Dam ase* is expressed in all neuroblasts except for NB 7-1 (Fig. 2E–H; Suppl. Table 1). Similar to *Dam sna* expression, the pattern of *Dam ase* positive neuroblasts is highly variable in individual hemi-neuromeres (Fig. 2E, F). *Dam pros* remains expressed in NB 1-1 but by the end of NS3, transcripts are up-regulated in addition in NBs 1-2, 3-1, 3-3, 4-3, 5-1, 5-2, 6-1, 6-2, 6–4, 7-2 and 7-4 (Fig. 1I–L; Suppl. Fig. 2A, B; Suppl. Table 1). At NS4, *Dam sna* is expressed in all neuroblasts taking into account all thoracic segments (Fig. 1M–P; Suppl. Fig. 2C–F). *Dam ase* is expressed in the remaining neuroblast that has not expressed *Dam ase* before (NB 7-1; Fig. 2I–L). At the same time *Dam pros* is expressed in neuroblasts of all rows and columns taking into account all thoracic segments (Fig. 1N–P; Suppl. Fig. 2C–F; Suppl. Table 1); however, the pattern of *Dam pros* positive neuroblasts is variable in each hemi-neuromere. By the end of NS4, the overall number of neuroblasts seems to decline (Suppl. Fig. 2E, F). In contrast to insects and malacostracan crustaceans (e.g., Bossing and Technau, 1994; Doe and Goodman, 1985a; Gerberding and Scholtz, 1999,2001), we did not detect a midline neuroblast.

Taken together, these data show that neuroblasts appear at fixed positions but at random sequence in the individual hemi-neuromeres of *D. magna*. Most neuroblasts are present at early neurogenesis but do not generate GMCs before late neurogenesis which accounts for a delay of about 10 h. We have identified 25 neuroblasts in *D. magna* which like in all other euarthropods are arranged in 7 rows indicating that there might be a developmental constraint on retaining this arrangement in all groups (Döffinger and Stollewerk, 2010).

Members of the Notch signalling pathway are expressed in neuroblasts

Next we analysed the expression patterns of members of the Notch signalling pathway. We identified single *Notch* (*Dam N*) and *Delta* (*Dam Dl*) homologues and three *Hairy/Enhancer of split* (*Hes*) orthologues, two of which – *Hes2* and *3* – are expressed during neurogenesis. Initially, all four genes are expressed in the central area of the hemi-neuromeres that does not generate neuroblasts but presumably gives rise to epidermis (Ungerer et al., 2011) (Fig. 4; Suppl. Fig. 4A, B, F, G, K, L, P, Q). Notch signalling seems to be active in the central area throughout neurogenesis since *Dam N*, *Dam Hes2* and *Hes3* remain expressed in this area (Suppl. Fig. 4H–J, M–O, R–S). In addition, all four genes are expressed in

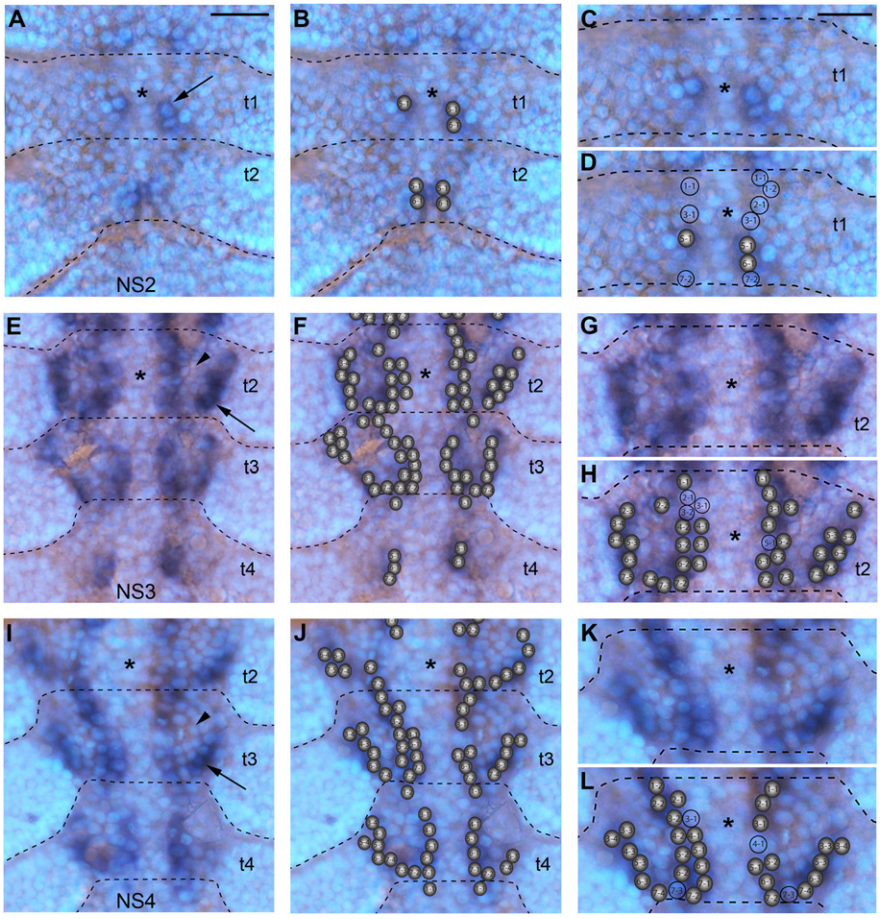

Fig. 2. (A–L) Sequence of *Dam asense* expression in neuroblasts. Fluorescent micrographs of flat preparations of *D. magna* embryos stained with a DIG-labelled RNA probe of *Dam ase* (dark blue) and the nuclei marker Hoechst (light blue); anterior is towards the top. The dashed horizontal lines indicate the segmental borders. The arrows point to neuroblasts; the arrowheads indicate the central area of the hemi-neuromeres that does not generate neuroblasts. The asterisks indicate the ventral midline. The images in the 1st column show *Dam ase* expression, the 2nd column shows the same sections with *Dam ase*+ neuroblasts labelled by grey spheres. The 3rd column shows magnifications of individual thoracic segments of the same embryos shown in the left columns. (A–D) At NS2, two neuroblasts in row 5 and 6 express *Dam ase* in the 1st and 2nd thoracic hemi-neuromeres. (E–H) At NS3, most neuroblasts express *Dam ase* in the thoracic segments. The arrangement in 7 rows is clearly visible. (I–L) In NS4, the number of neuroblasts expressing *Dam ase* decreases in individual hemi-neuromeres. *t1 to t4*, thoracic segments 1 to 4. Scale bar: (A) 50 μm in A, B, E, F, I, J; (C) 25 μm in C, D, G, H, K, L. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

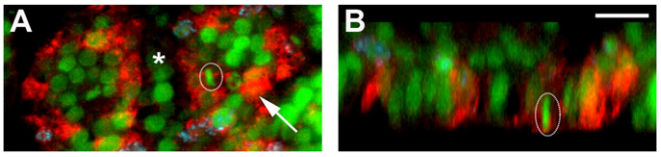

Fig. 3. (A–B) Symmetric division of *Daphnia magna* neuroblast. Confocal micrographs of *D. magna* embryo stained with a fluorescein-labelled RNA probe of *Dam sna* (red) and Hoechst (green) for nuclei; anterior is towards the top in A and dorsal towards the top in B. Asterisks indicate the ventral midline. (A, B) Horizontal and transverse sections of the VNE showing the symmetric division of a *Dam sna* positive neuroblast (metaphase plate encircled) in the plane of the VNE at stage NS3. (B) 20 μm in A, B. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

neuroblasts (Fig. 5; Suppl. Fig. 4). *Dam Dl* expression is switched on in neuroblasts shortly after their formation and resembles *ASH* expression (Figs. 5G, H; 6N; Suppl. Fig. 4A–E) (Ungerer et al., 2011), while *Dam Hes2* and *Hes3* are expressed in subsets of neuroblasts at a given time (Fig. 5B–E; Suppl. Fig. 4L, M, Q, R). During NS4, *Dam Hes2* and *Hes3* expression becomes restricted to the central domains that do not generate neuroblasts (Suppl. Fig. 4N, S). *Dam N* is expressed ubiquitously but transcripts are up-regulated strongly in neuroblasts in NS3 to NS5 (Fig. 5I, J; Suppl. Fig. 4).

Inactivation of Notch signalling results in premature generation of GMCs

We inhibited Notch activation using the γ-secretase inhibitor DAPT (Geling et al., 2002; Pueyo et al., 2008). In all affected embryos, the overall morphology was changed due to defects in segmentation, limb formation and deformation of the ventral midline (Fig. 6; Suppl. Figs. 5–7). Inactivation of Notch signalling results in reduced expression of *Dam Hes2* and *Hes3* suggesting that these genes are targets of the Notch signalling pathway (Fig. 6A, B; Suppl. Fig. 5).

Next we analysed the expression pattern of *Dam sna*—the earliest gene expressed in neuroblasts. In DAPT embryos *Dam sna* is expressed at high levels in all cells of the VNE throughout neurogenesis (Fig. 6C, G–I; Suppl. Fig. 6). This includes the area of neuroblast formation which is seen as a ring-like structure in the hemi-neuromeres of control embryos as well as the central area of the hemi-neuromeres that does not give rise to neuroblasts and lacks *Dam snail* expression in the control (Fig. 6H, I). The *Dam sna* positive cells in the VNE of DAPT embryos vary in size (Fig. 6C, H; Suppl. Fig. 6). Based on the fact that in untreated embryos *Dam sna* is expressed in neuroblasts and transiently in GMCs, which are smaller than neuroblasts (Suppl. Fig. 3), we assume that the cells in the neuroectoderm of DAPT embryos consist of a mixture

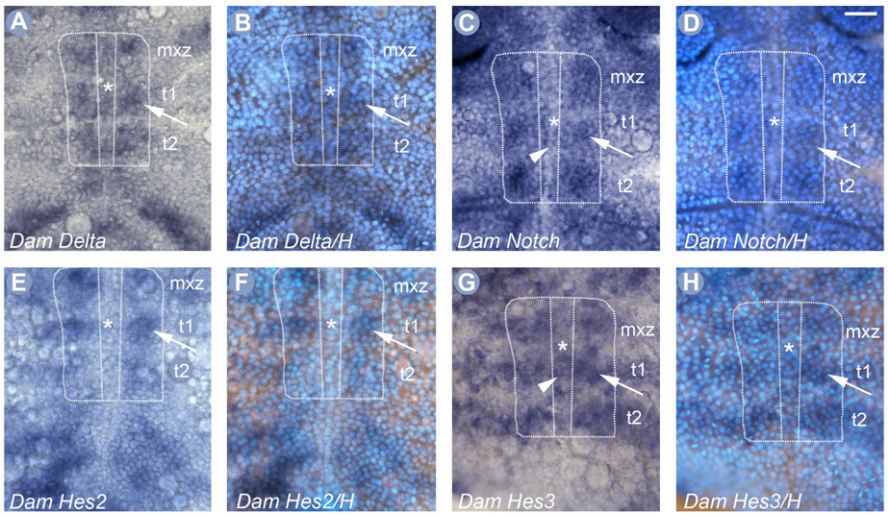

Fig. 4. (A–H) Expression patterns of members of the Notch signalling pathway in the central area at the beginning of neurogenesis. Light and fluorescence micrographs of *D. magna* embryos (stage NS1) stained with DIG-labelled RNA probes of *Dam Dl, Dam N, Dam Hes2* and *Hes3* (dark blue) and Hoechst (light blue); anterior is towards the top. The same sections are shown for the expression patterns of the individual genes with and without Hoechst (H) staining. Encircled area: VNE of the posterior maxillary zone and the 1st and 2nd thoracic segment. The asterisks indicate the ventral midline; the arrows indicate the expression in the central area of the hemi-neuromeres. The segmental furrows have not formed at NS1 and it is therefore not possible to determine if the central expression corresponds exclusively to the area of the hemi-neuromeres that does not generate neuroblasts at this stage. (A, B) *Dam Dl* is expressed in all VNE cells and shows a strong expression in the central area. (C, D) *Dam N* is expressed throughout the VNE but shows a stronger expression in the central area. The arrowhead points to *Dam N* expression in ventral midline cells. (E–H) The Notch effector genes *Dam Hes2* and *3* are strongly expressed in the central area. *Dam Hes3* is expressed in ventral midline cells (arrowhead). *mxz*, maxillary zone; *t1 to t2*, thoracic segments1 to 2. Scale bar: (D) 50 μm in A–H. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

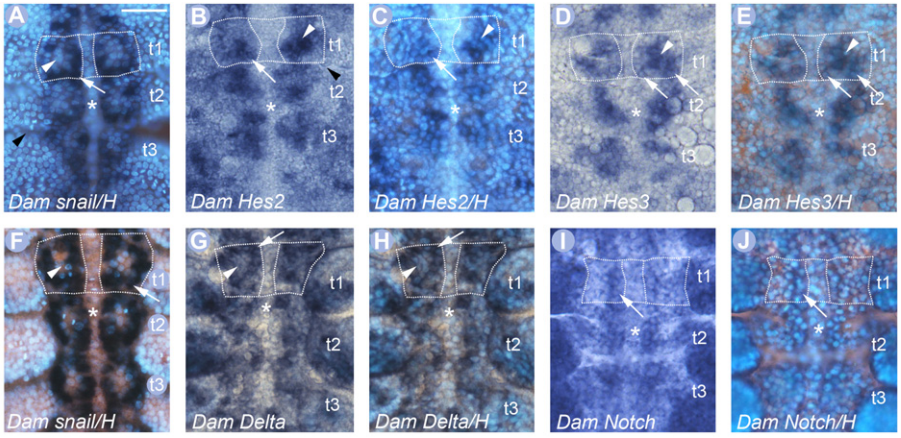

Fig. 5. (A–J) Expression pattern of members of the Notch signalling pathway. Light and fluorescence micrographs of *D. magna* embryos stained with DIG-labelled RNA probes of *Dam sna*, *Dam Dl, Dam N, Dam Hes2* and *Hes3* (dark blue) and Hoechst (light blue); anterior is towards the top. The same sections are shown for the expression patterns of the individual genes with and without Hoechst (H) staining except for *Dam sna*. Encircled areas: thoracic neuromere 1. The arrows point to neuroblasts; the arrowheads indicate expression in the central area of the hemi-neuromeres. Asterisks: ventral midline. The figure shows ventral views of the thoracic neuroectoderm. In a given hemi-neuromere, the staining in the central area of the VNE corresponds to the region that does not generate neuroblasts, while the surrounding ring-like area corresponds to the region where neuroblasts are formed. (A) At stage NS2 the segmental borders appear (black arrowhead) and the ring-like arrangement of *Dam sna* expressing neuroblasts becomes visible in the thoracic hemi-neuromeres. (B, C) The fixed arrangement of neuroblasts as well as the limited number of cells within a hemi-neuromere and clear landmarks (towards medial the ventral midline, towards lateral the limb buds and towards anterior and posterior the intersegmental furrows (black arrowhead) allow for identifying VNE cells as neuroblasts. The arrow points to a posterior-medial *Dam Hes2* expressing neuroblast (NB 7-2) at NS3. *Dam Hes2* remains expressed in the central area (arrowhead). (D, E) Stage NS2; *Dam Hes3* is expressed in neuroblasts. The arrows point to lateral and medial neuroblasts. *Dam Hes3* is also expressed in the central area (arrowhead). (F) Stage NS4; the neuroblasts strongly express *Dam sna*. (G, H) Stage NS4; *Dam Dl* is up-regulated in neuroblasts (arrow). Like *Dam ASH* (Fig. 6N), *Dam Dl* is expressed at various levels in neuroblasts. (I, J) *Dam N* is expressed in all VNE cells but shows a stronger expression in subsets of neuroblasts. The arrows point to the medial column of neuroblasts that show strong expression of *Dam N* at NS4. *t1 to t3*, thoracic segments1 to 3. Scale bar: (A) 50 μm in A–J. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

of neuroblasts and GMCs. This is supported by the same over-expression of *Dam pros* in all cells of the VNE in DAPT embryos (Fig. 6D, E, J, K; Suppl. Fig. 7). In control embryos *Dam pros* is exclusively expressed in neuroblasts generating GMCs and the GMCs themselves (Fig. 6F, L; Suppl. Fig. 7D, H, L, P, T). The VNE phenotype can already be detected in NS2 indicating that neuroblasts divide immediately after their formation (Suppl. Fig. 7A–C). The presence of GMCs within the VNE of DAPT embryos suggests that their premature formation prevents the ordered formation of basal layers.

The premature presence of GMCs in DAPT embryos indicates that neuroblasts start dividing earlier. We compared the pattern of mitotic divisions in DAPT and control embryos by using the mitotic marker anti-Phospho-Histone 3 (Fig. 7). In control embryos the delay between neuroblast formation and division is reflected in the near absence of cell divisions in NS2 (Fig. 7A, B)

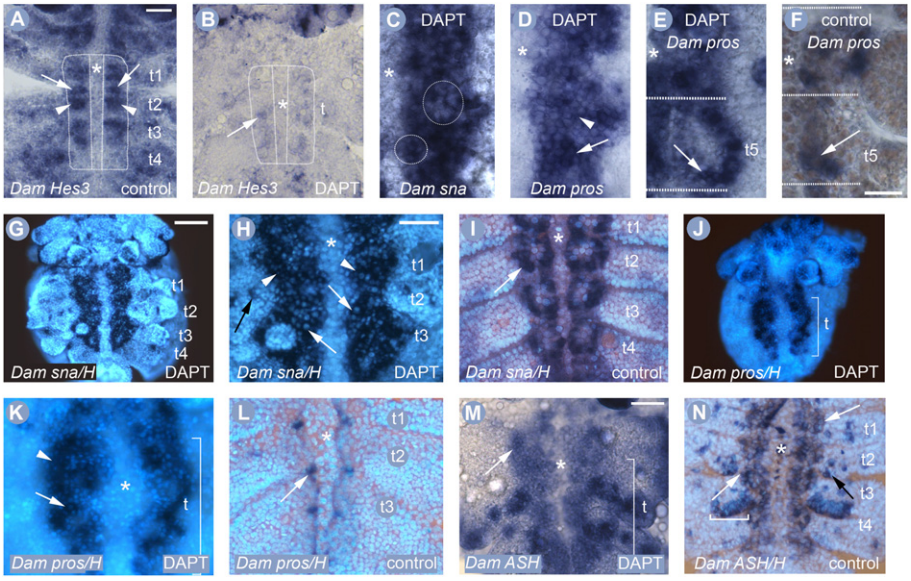

Fig. 6. (A–N) Analysis of Notch function in *D. magna* neurogenesis. Light and fluorescence micrographs of *D. magna* embryos stained with DIG-labelled RNA probes of *Dam Hes3, Dam sna*, *Dam pros, Dam ASH* (dark blue) and Hoechst (light blue); anterior is towards the top. Sections C–F show high magnifications of thoracic hemi-neuromeres; the ventral midline is towards the left (asterisks). Asterisks indicate the ventral midline. We treated 451 embryos with DAPT. 39% of the embryos were not analysable because they were either too young or too old. 63% of the remaining DAPT embryos showed a specific phenotype as revealed by morphology and changes in the expression patterns of *Dam Hes2*, *Dam Hes3*, *Dam sna*, *Dam pros* and *Dam ASH*. (A) At NS2 *Dam Hes3* is expressed in neuroblasts (arrows) and the central domains (arrowheads) in control embryos. (B) Stage NS2; *Dam Hes3* expression is reduced in DAPT embryos; the arrow points to residual expression. (C) Stage NS3; all VNE cells express *Dam sna* in DAPT embryos. The cells vary in size. In this section a group of small cells and a group of large cells is visible (compare large and small encircled areas). (D) *Dam pros* expression in a DAPT embryo at NS2. Neuroblasts (arrow) are present in the central domain (arrowhead). (E, F) Stage NS4; GMCs (arrow in E) are visible in DAPT embryos in positions where neuroblasts are present in the control (arrow in F). (G) Overview of *Dam sna* expression in a DAPT embryo at NS3. *Dam sna* is expressed in all VNE cells. (H) Magnification of thoracic neuromeres of the same DAPT embryo shown in G. The arrowheads point to small cells (presumptive GMCs) and the arrows to large cells (presumptive neuroblasts). *Dam sna* is prematurely expressed in sensory precursor cells in the limb anlagen (black arrow). See also Suppl. Figs. 6 and 7 for expression of *Dam sna* and *pros* in sensory precursors. (I) *Dam sna* expression in a control embryo at NS3. The arrow points to neuroblasts. (J) Overview of *Dam pros* expression in a DAPT embryo at NS2/3. *Dam pros* is expressed in all VNE cells. (K) Magnification of thoracic neuromeres of the same DAPT embryo shown in J. The arrowheads point to small cells (presumptive GMCs) and the arrows to large cells (presumptive neuroblasts). (L) Expression pattern of *Dam pros* in a control embryo at NS2. The arrow points to a neuroblast. (M) *Dam ASH* is strongly expressed in all VNE cells in DAPT embryos at NS4. (N) 'Salt and pepper' expression pattern of *Dam ASH* in a control embryo at NS4. The bracket indicates the expression of *Dam ASH* in clusters of sensory precursors in the limb anlagen. *Dam ASH* is also expressed in scattered sensory precursors (black arrow). *t*, thoracic area; *t1 to t4*, thoracic segments1 to 4. Scale bars: (A) 50 μm in A,B; (F) 20 μm in C–F; (G) 100 μm in G, J; (H) 50 μm in H, I, K, L; (M) 50 μm in M, N. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

(Ungerer et al., 2011). A few mitotic cells can be detected in NS3 but most divisions occur in NS4 in control embryos (Fig. 7E, F, I, J). In contrast, there is an earlier onset of mitotic divisions in DAPT embryos in NS2 and many divisions can be detected at NS3 (Fig. 7C, D, G, H). These data confirm the assumption that neuroblasts divide prematurely in DAPT embryos.

The fact that *Dam pros* is not expressed in the same pattern as the Notch effector genes *Dam Hes2* and *Hes3* in untreated embryos implies an indirect regulation of *Dam pros* by Notch signalling. Since experiments in mammalian neural stem cells show that MASH1 induces *Prox1* expression (Torii et al., 1999), we analysed whether *Dam ASH* expression is changed in DAPT embryos. We found that *ASH* shows a strong homogenous expression in the whole VNE in severely affected DAPT embryos, while *ASH* is expressed at different levels ('salt and pepper' pattern) in neuroblasts of control embryos (Fig. 6M, N). This data suggests that high levels of *Dam ASH* switch on *Dam pros* expression which in turn leads to premature asymmetric division of neuroblasts and GMC production.

Discussion

We show here that Notch signalling has two functions in early neurogenesis in the branchiopod crustacean *D. magna*: it is required for binary cell fate decisions in the VNE and for controlling the time of GMC production. These functions are only partially comparable to the role of Notch signalling in the corresponding processes in insects. In the following, we compare the various roles of Notch signalling in arthropod neurogenesis and discuss how the pathway might have been co-opted to the development of the evolving neuroblasts.

The role of Notch signalling in GMC production

In the insect VNE, Notch signalling is exclusively associated with binary cell fate decisions (Heitzler and Simpson, 1991). Proneural genes confer neural potential to clusters of neuroectodermal cells (proneural clusters) and Notch activity represses proneural gene expression in all but one cell of the cluster, the nascent neuroblast. Neuroblasts delaminate within minutes of their selection and immediately divide producing GMCs (Hartenstein et al., 1992). The spatio-temporal regulation of proneural gene expression determines the time and position of neuroblast formation which in turn determines the production of GMCs due to the conjunction of delamination and division (Skeath et al., 1992). This mode of neurogenesis does not require the regulation of different neuroblast states in the VNE by Notch signalling. In contrast in the crustacean, neuroblasts are not selected from proneural clusters; the *achaete-scute homologue* is only expressed after formation of the neuroblasts. Furthermore, neuroblast formation is not associated with delamination and division as in *Drosophila* (Ungerer et al., 2011). Notch signalling is required to keep neuroblasts in a transitory state by suppressing *Dam pros* expression thereby preventing premature

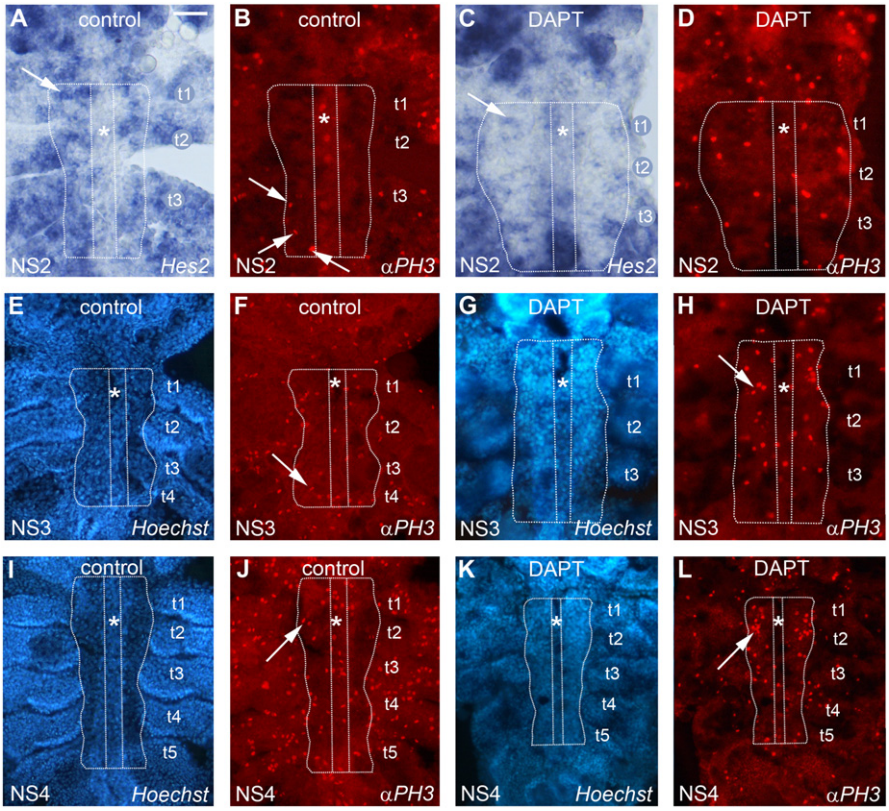

Fig. 7. Early onset of cell proliferation in the VNE of DAPT embryos. Light and fluorescence micrographs of flat preparations of *D. magna* embryos stained with DIG-labelled RNA probes of *Dam Hes2* (dark blue), anti-Phospo Histone 3 (α-PH3; red) and Hoechst (light blue); anterior is towards the top. *Dam Hes2* staining was used in addition to morphology to detect the reduction in Notch signalling and is shown for stage NS2. The dashed outline indicates the area of the VNE and the ventral midline (asterisks). (A, B) Same section of a control embryo at NS2; *Dam Hes2* expression is up-regulated in scattered neuroblasts (arrow in A). At this stage, cell divisions are almost absent. Only 3 mitotic cells are visible in the VNE of the embryo presented here (arrows in B). (C, D) Same section of a DAPT embryo at NS2; *Dam Hes2* expression is reduced. Weak expression is seen in some neuroblasts (arrow in C). Note the expansion of the VNE in this embryo. Many proliferating cells are present in the VNE (encircled area in D). (E, F, I, J) α–PH3 staining in NS3 and NS4 control embryos. There is a peak of α-PH3 staining at the time when most neuroblasts express *Dam pros* and divide at NS4 (J). (G, H, K, L) At NS3, DAPT embryos show a considerably higher expression of α-PH3 in the VNE as compared to the control, while the pattern is more similar to the respective control embryos in NS4. *t1 to t5*, thoracic segments 1 to 5. Scale bar: (A) 50 μm in A–L. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

GMC production and differentiation. A similar phenotype has been described in chick and mouse embryos where inactivation of Notch signalling in neural progenitor cells leads to significant induction of *Prox1* expression which in turn results in premature production of neurons (Kaltezioti et al., 2010).

Further comparison of crustacean and vertebrate neurogenesis reveals that mechanisms of neuroblast regulation have evolved in these long-diverged groups that share surprising similarities. Like in vertebrates, crustacean neural stem cells are neuroepithelial cells that remain in the epithelium when producing neural precursors (Dohle, 1976; Scholtz, 1992; Ungerer and Scholtz, 2008). Furthermore, crustacean neuroblasts divide symmetrically in the VNE to produce ectodermal cells that can generate neuroblasts as has been shown in malacostracans (Dohle, 1976; Ungerer, 2006). Symmetric divisions of *Dam sna+* neuroblasts in *D. magna* suggest similar mechanisms in branchiopods.

The similarity in the morphogenetic processes seems to be supported by the same molecular interactions of the members of the Notch signalling pathway and the proneural genes. Our functional and expression studies suggest that *Dam pros* is indirectly regulated by Notch signalling via *Dam ASH*, similar to the case in vertebrates. In untreated embryos *Dam ASH* is expressed in a 'salt and pepper' pattern in the ring-like VNE domains indicating different levels of expression in individual neuroblasts. Recently, a 'salt and pepper' expression pattern of the proneural gene *Neurogenin2* has been described in the developing mammalian brain and was shown to result from oscillation of *Neurogenin2* and *Hes1* (Kageyama et al., 2009). *Hes1* is activated in neural stem cells by Notch but is repressed by its own gene product. In cells with active Notch signalling, this leads to oscillation of Hes1 in neural stem cells and in turn to oscillation of *Neurogenin2* (proneural gene) which is periodically repressed by Hes1. During oscillation neural genes that control asymmetric cell division such as *Prox1* cannot be switched on because they seem to require prolonged high expression levels of the proneural gene (Kageyama et al., 2009). Thus neural precursor production can only occur after Notch signalling has been switched off. The mechanism establishes a balance between stem cell production and generation of neural precursors. Our functional and gene expression data suggest that this model can be applied to *D. magna* neurogenesis indicating that Notch function has evolved in a similar way in crustaceans and vertebrates (Fig. 8A).

Interestingly, Notch signalling controls the differentiation of neuroblasts in some areas of the *Drosophila* brain although the molecular and/or morphological mechanisms are different compared to the VNE of crustaceans. In *Drosophila* the optic lobes develop from a bilateral neuroepithelium in the larva. Transcripts of the proneural gene *lethal of scute* are up-regulated in a wave-like pattern that sweeps across the neuroepthelium from medial to lateral (Yasugi et al., 2008). Neuroepithelial cells with *lethal of scute* expression are transformed into neuroblasts which delaminate. Egger et al. (2010) could show that Notch is required for

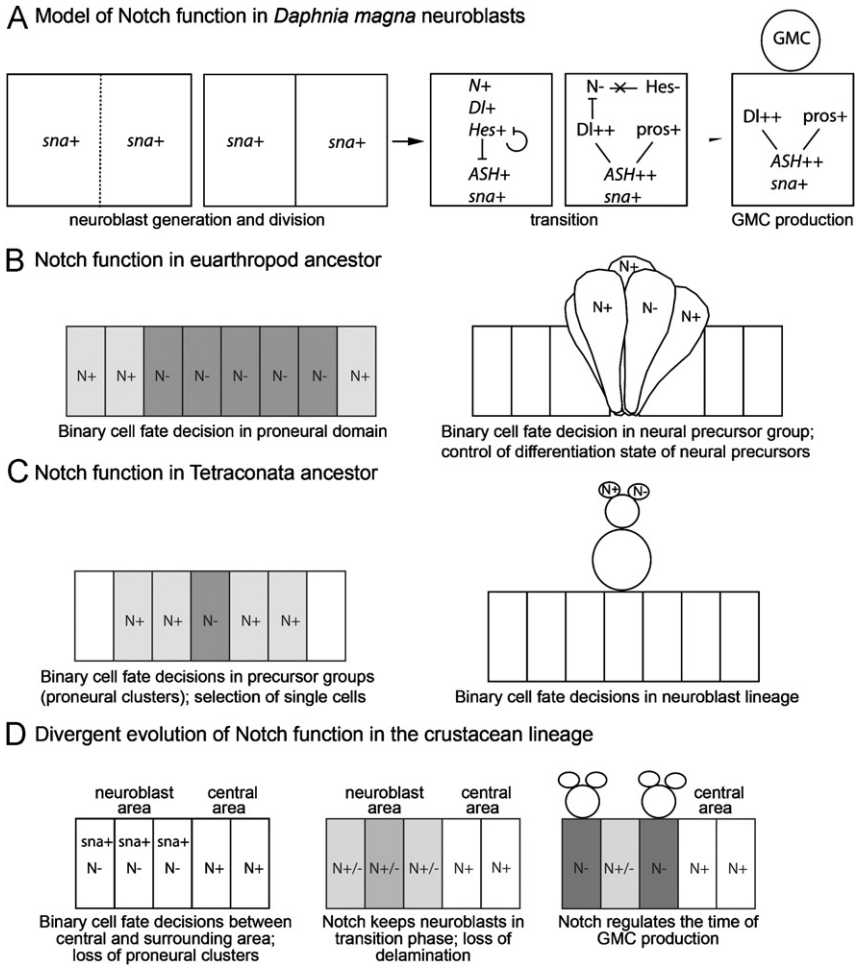

Fig. 8. (A–D) Model of neuroblast regulation in *D. magna* and evolution of Notch function. (A) The model is based on the mammalian model for neural stem cell regulation. In *D. magna, sna* positive neuroblasts are generated and some of them divide symmetrically within the VNE. In the transition phase leading to GMC production, *Dam ASH*, *Dam Dl*, *Dam N* and *Dam Hes2* and *3* are expressed in neuroblasts. *Dam Hes2* and *3* repress *Dam ASH* and also their own expression. For as long as Notch is active in the neuroblast, *Dam Hes2* and *3* expression will be switched on again resulting in cyclic expression of *Dam Hes2, Dam Hes3* and *Dam ASH*. Slightly higher levels of *Dam ASH* accumulate over time in individual neuroblasts which in turn results in higher levels of *Dam Dl*. Notch signalling is inhibited and the sustained high levels of *Dam ASH* lead to activation of *Dam pros* and transition to the GMC production phase. (B) Notch signalling has an ancestral function in binary cell fate decisions and controlling the differentiation of precursors by keeping them in an undifferentiated state in the epithelium. (C) In the Tetraconata ancestor Notch signalling is co-opted to neuroblast development keeping cells in the epithelium that are not selected for the neural fate. (D) Changes in the selection mechanisms of neuroblasts are accompanied by an additional function of Notch in controlling the transition state of neuroblasts.

maintaining the neuroepithelial state. In Notch mutant clones, neuroblasts form prematurely and delaminate. This in turn leads to premature differentiation of the optic lobes.

The role of Notch signalling in binary cell fate decisions

Notch signalling is furthermore required for binary cell fate decisions in the VNE of *D. magna*. In contrast to insects the decision does not occur within proneural clusters but between the cells in the central domain of the hemi-neuromeres and the surrounding cells. Before formation of the neuroblasts, *Dam Delta* is expressed at low levels in the neuroectoderm including the central domain. The Notch receptor is activated in the central domain as reflected by the expression of the effector genes *Hes2* and *Hes3*. This restricts the formation of neuroblasts to a ring-like domain in each hemi-segment. The spatial subdivision of epidermal and neural progenitors into a central and peripheral domain in each hemi-neuromere seems to be representative for branchiopods; however, distinct mechanisms seem to operate in malacostracans, the only other crustacean group that has been studied (Harzsch, 2001; Scholtz, 1992; Wheeler and Skeath, 2005).

It is interesting to note that Notch function in binary cell fate decisions in the VNE leads to different outcomes in the various euarthropod groups indicating that Notch signalling has been modified during evolution of the individual lineages. In the remaining euarthropod groups, the chelicerates and myriapods, groups of neural precursors are selected for the neural fate which represents the ancestral pattern of neurogenesis (Dove and Stollewerk, 2003; Stollewerk et al., 2001). Loss of function of either Notch or Delta in the spider (chelicerate) 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* (Stollewerk, 2002). Thus, although Notch signalling appears to be used in the same way as in *Drosophila*, the lateral inhibition process produces groups of neural precursors, rather than single neuroblasts. These data contradict the lateral inhibition model in which a cell that has taken on the neural fate inhibits its immediate neighbours from adopting the same fate (Simpson, 1990). However, studies in the *Drosophila* eye and wing disc have shown that the outcome of Notch signalling depends on the ratio of Delta to Notch within a cell. Cis-inhibition of the Notch receptor by the Delta ligand, makes cells insensitive to Notch signalling and thus influences the

directionality of Notch signalling (reviewed by del Alamo et al., 2011). This mechanism explains how adjacent cells can adopt the same fate and how various patterns of binary cell fates can be generated. Interestingly, cis-inhibition does neither play a major role in the spaced arrangement of sensory organ precursors nor of neuroblasts in the VNE of *Drosophila* since the pattern is robust over a wide range of Delta concentrations (Brennan et al., 1997; Doherty et al., 1997; Li and Baker, 2004; Seugnet et al., 1997). Thus, the presence or absence of cis-inhibition might determine if neural progenitors are spaced (insects) or are formed next to each other (branchiopods, chelicerates, myriapods). In addition, pre-patterning mechanism as well as numerous modifiers of Notch signalling (e.g., Neuralized, Mind Bomb, members of the Bearded family, micro-RNAs, Numb) are likely to contribute to the precise localisation of Notch activity (reviewed by Andersson et al., 2011).

How has Notch signalling been co-opted to neural stem cell development?

Due to the independent origin of crustacean and vertebrate neural stem cells, it can be assumed that Notch signalling has been incorporated into controlling stem cell development in these groups by different ways. However, the data on early neurogenesis are fragmentary in basal chordates and closely related groups such as hemichordates and echinoderms (Holland et al., 2001,2000; Rasmussen et al., 2007) and it is therefore not clear at which point of chordate evolution the complex features of neural stem cells evolved and came under the control of Notch signalling.

In arthropods the function of Notch in binary cell fate decisions and in controlling the differentiation of neural precursors predates the evolution of neuroblasts and was at least present in the last common ancestor of euarthropods (Fig. 8B). The ancestral pattern of euarthropod neurogenesis is the formation of neural precursor groups that directly differentiate into neural cells. This mode of neurogenesis has been retained in myriapods and chelicerates (Chipman and Stollewerk, 2006; Dove and Stollewerk, 2003; Stollewerk et al., 2001). Notch signalling regulates binary cell fate decisions in the VNE by restricting proneural gene expression to spaced groups of neural precursors (Stollewerk, 2002). Furthermore, Notch signalling is active in the neural precursor groups after their formation (Fig. 8B). Detailed analysis in the peripheral nervous system of a spider (chelicerate) showed that Notch signalling has two functions in the precursor groups: it regulates binary cell fate decisions between two cell populations and it maintains the precursors in an epithelial, undifferentiated state (Gold et al., 2009). Numb acts as antagonist of Notch signalling and promotes the neural fate in the sensory precursor groups. In contrast to *Drosophila*, binary cell fate decisions are not coupled with asymmetric cell divisions in the spider: rather, cell fate determinants such as Prospero are expressed de novo in neural precursors (Gold et al., 2009; Weller and Tautz, 2003). Loss of Notch function results in disintegration of the precursor groups and the absence of sense organs, while a reduction in Notch activity leads to additional cells adopting the neural fate and premature differentiation (Gold et al., 2009).

We suggest that the function of Notch signalling in maintaining cells in an epithelial state and controlling binary cell fate decisions has culminated in singling out individual cells from the precursor groups. This was probably facilitated by parallel changes in the regulatory regions of the proneural genes (Ayyar et al., 2010; Eriksson and Stollewerk, 2010). Since the ancestral state of neurogenesis in arthropods is the segregation of neural precursors, we assume that the same mechanism was adopted for neuroblasts in the Tetraconata ancestor. The ancestral function of Notch in maintaining precursor cells in an epithelial state was possibly co-opted for retaining cells that were not selected for the neural fate in the VNE (Fig. 8C). This mode of neurogensis was maintained in insects. The gradual integration of cell division and asymmetric distribution of neural cell fate determinants might have facilitated the evolution of neural stem cells in the last common ancestor of Tetraconata.

In the crustacean lineage Notch signalling has adopted a different role. A temporal shift of *sna* (earlier) and *ASH* (later) expression is accompanied by a change in neuroblast selection and the evolution of mechanisms similar to vertebrate neural stem cell regulation. Neuroblasts are not selected from proneural clusters but appear next to each other in defined positions. Like vertebrate neural stem cells, crustacean neuroblasts are kept in the VNE. By maintaining neuroblasts in a transitory state, Notch signalling regulates the time of GMC production and thus the differentiation of the nervous system (Fig. 8D).

Conclusion

Our data suggest that the Notch signalling pathway has been co-opted from pre-existing structures – the neural precursor groups – to the development of the evolving neural stem cells in the Tetraconata ancestor. The function of Notch signalling has diverged in insects and crustaceans accompanied by changes in the morphogenetic processes. In the crustacean, Notch controlled mechanisms of neuroblast regulation have evolved that are surprisingly similar to vertebrates. This presents a remarkable case of parallel evolution which does not only involve the co-option of a conserved signalling pathway but also the independent evolution of the same genetic interactions and similar morphogenetic processes.

Database link

GenBank: Genetic sequence database at the National Centre for Biotechnology Information (NCBI) (GenBank IDs: HQ259915, *Dam Dl*; HQ398106, *Dam N*; JN679203, *Dam Hes1*; JN679204, *Dam Hes2*; JN679205, *Dam Hes3*).

Acknowledgements

We are grateful to Dieter Ebert for helping us establishing the *D. magna* culture and giving advice and comments on the project. We also thank Dieter Ebert for sharing unpublished *D. magna* sequences. Thanks to Jürgen Hottinger from the Ebert lab for technical support. We are grateful to Jean-Claude Walser for identifying the *Dam Hes* genes in the unpublished *D. magna* genome. Thanks to Edina Balczo for technical support. This work was supported by Grants Un 271/1-1 (German Research foundation) to PU and BB/F021909/1 (Biotechnology and Biological Sciences Research Council) to AS.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2012.08.025.

References

Andersson, E.R., Sandberg, R., Lendahl, U., 2011. Notch signalling: simplicity in design, versatility in function. Development 138, 3593–3612.

Ayyar, S., Negre, B., Simpson, P., Stollewerk, A., 2010. An arthropod cis-regulatory element functioning in sensory organ precursor development dates back to the Cambrian. BMC Biol. 8, 127.
Bate, M., 1976. Embryogenesis of an insect nervous system. I. A map of thoracic and abdominal neuroblasts in *Locusta migratoria*. J. Embryo. Exp. Morphol. 35, 107–123.
Bossing, T., Technau, G.M., 1994. The fate of the CNS midline progenitors in *Drosophila* as revealed by a new method for single cell labelling. Development 120, 1895–1906.
Bossing, T., Udolph, G., Doe, C.Q., Technau, G.M., 1996. The embryonic central nervous system lineages of *Drosophila melanogaster*. I. Neuroblast lineages derived from the ventral half of the neuroectoderm. Dev. Biol. 179, 41–64.
Brennan, K., Tateson, R., Lewis, K., Arias, A.M., 1997. A functional analysis of Notch mutations in *Drosophila*. Genetics 147, 177–188.
Campos-Ortega, J.A., 1993. Early neurogenesis in *Drosophila melanogaster*. In: Bate, M., Arias, A. (Eds.), The Development of *Drosophila melanogaster*, vol. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp. 1091–1129.
Chipman, A.D., Stollewerk, A., 2006. Specification of neural precursor identity in the geophilomorph centipede *Strigamia maritima*. Dev. Biol. 290, 337–350.
del Alamo, D., Rouault, H., Schweisguth, F., 2011. Mechanisms and significance of *cis*-Inhibition in Notch Signalling. Curr. Biol. 21, R40–R47.
Doe, C.Q., Goodman, C.S., 1985a. Early events in insect neurogenesis. I. Development and segmental differences in the pattern of neuronal precursor cells. Dev. Biol. 111, 193–205.
Doe, C.Q., Goodman, C.S., 1985b. Early events in insect neurogenesis. II. The role of cell interactions and cell lineage in the determination of neuronal precursor cells. Dev. Biol. 111, 206–219.
Döffinger, C., Stollewerk, A., 2010. How can conserved gene expression allow for variation? Lessons from the dorso-ventral patterning gene *muscle segment homeobox*. Dev. Biol. 345, 105–116.
Doherty, D., Jan, L.Y., Jan, Y.N., 1997. The Drosophila neurogenic gene big brain, which encodes a membrane-associated protein, acts cell autonomously and can act synergistically with Notch and Delta. Development 124, 3881–3893.
Dohle, W., 1976. Die Bildung und Differenzierung des postnauplialen Keimstreifs von *Diastylis rathkei* (Crustacea, Cumacea) II. Die Differenzierung und Musterbildung des Ektoderms. Zoomorph 84, 235–277.
Dove, H., Stollewerk, A., 2003. Comparative analysis of neurogenesis in the myriapod *Glomeris marginata* (Diplopoda) suggests more similarities to chelicerates than to insects. Development 130, 2161–2171.
Egger, B., Gold, K.S., Brand, A.H., 2010. Notch regulates the switch from symmetric to asymmetric neural stem cell division in the *Drosophila* optic lobe. Development 137, 2981–2987.
Eriksson, B.J., Stollewerk, A., 2010. Expression patterns of neural genes in *Euperipatoides kanangrensis* suggest divergent evolution of onychophoran and euarthropod neurogenesis. Proc. Natl. Acad. Sci. USA 107, 22576–22581.
Geling, A., Steiner, H., Willem, M., Bally-Cuif, L., Haass, C., 2002. A gamma-secretase inhibitor blocks Notch signalling in vivo and causes a severe neurogenic phenotype in zebrafish. EMBO Rep. 3, 688–694.
Gerberding, M., Scholtz, G., 1999. Cell lineage of the midline cells in the amphipod crustacean *Orchestia cavimana* (Crustacea, Malacostraca) during formation and separation of the germ band. Dev. Genes Evol. 209, 91–102.
Gerberding, M., Scholtz, G., 2001. Neurons and glia in the midline of the higher crustacean *Orchestia cavimana* are generated via an invariant cell lineage that comprises a median neuroblast and glial progenitors. Dev. Biol. 235, 397–409.
Gilbert, S.F., 2006. Developmental Biology. Sinauer Associates, Sunderland.
Giribet, G., Egdecombe, G.D., 2011. Reevaluating the arthropod tree of life. Annu. Rev. Entomol. 57, 167–186.
Gold, K., Cotton, J.A., Stollewerk, A., 2009. The role of Notch signalling and *numb* function in mechansensory organ formtation in the spider *Cupiennius salei*. Dev. Biol. 327, 121–131.
Goodman, C.S., Doe, C.Q., 1993. Embryonic development of the *Drosophila* central nervous system. In: Bate, M., Martinez-Arias, A. (Eds.), The Development of *Drosophila melanogaster*. Cold Spring Harbor Laboratory Press, New York, pp. 1131–1206.
Hartenstein, A.Y., Rugendorff, A., Tepass, U., Hartenstein, V., 1992. The function of the neurogenic genes during epithelial development in the *Drosophila* embryo. Development 116, 1203–1220.
Harzsch, S., 2001. Neurogenesis in the crustacean ventral nerve cord: homology of neuronal stem cells in Malacostraca and Branchiopoda? Evol. Dev. 3, 154–169.
Heitzler, P., Simpson, P., 1991. The choice of cell fate in the epidermis of *Drosophila*. Cell 64, 1083–1092.
Holland, L.Z., Rached, L.A., Tamme, R., Holland, N.D., Inoko, H., Shiina, T., Burgtorf, C., Lardelli, M., 2001. Characterization and developmental expression of the Amphioxus Homolog of *Notch* (*AmphiNotch*): evolutionary conservation of multiple expression domains in Amphioxus and Vertebrates. Dev. Biol. 232, 493–507.
Holland, L.Z., Schubert, M., Holland, N.D., Neuman, T., 2000. Evolutionary conservation of the presumptive neural plate markers AmphiSox1/2/3 and *AmphiNeurogenin* in the invertebrate chordate Amphioxus. Dev. Biol. 226, 18–33.
Kageyama, R., Ohtsuka, T., Shimojo, H., Imayoshi, I., 2009. Dynamic regulation of Notch signaling in neural progenitor cells. Curr. Opin. Cell Biol. 21, 733–740.
Kaltezioti, V., Kouroupi, G., Oikonomaki, M., Mantouvalou, E., Stergiopoulos, A., Charonis, A., Rohrer, H., Matsas, R., Politis, P.K., 2010. Prox1 regulates the Notch-1-mediated inhibition of neurogenesis. PLoS ONE 8, e10000565, http://dx.doi.org/10.1371/journal.pbio.1000565.
Landgraf, M., Bossing, T., Technau, G.M., Bate, M., 1997. The origin, location, and projection of the embryonic abdominal motoneurons of *Drosophila*. J. Neurosci. 17, 9642–9655.
Li, Y., Baker, N.E., 2004. The roles of cis-inactivation by Notch ligands and of neuralized during eye and bristle patterning in Drosophila. BMC Dev. Biol. 4, 5.
Mayer, G., Whitington, P.M., 2009. Velvet worm development links myriapods with chelicerates. Proc. Biol. Sci. 276, 3571–3579.
Pueyo, J.I., Lanfear, R., Couso, J.P., 2008. Ancestral Notch-mediated segmentation revealed in the cockroach *Periplaneta americana*. Proc. Natl. Acad. Sci. USA 105, 16614–16619.
Rasmussen, S.L.K., Holland, L.Z., Schubert, M., Beaster-Jones, L., Holland, N.D., 2007. Amphioxus AmphiDelta: evolution of delta protein structure, segmentation and neurogenesis. Genesis 45, 113–122.
Regier, J.C., Shultz, J.W., Zwick, A., Hussey, A., Ball, B., Wetzer, R., Martin, J.W., Cunningham, C.W., 2010. Arthropod relationships revealed by phylogenomic analysis of nuclear protein-coding sequence. Nature 463, 1079–1084.
Schmidt, H., Rickert, C., Bossing, T., Vef, O., Urban, J., Technau, G.M., 1997. The embryonic central nervous system lineages of *Drosophila melanogaster*. II. Neuroblast lineages derived from the dorsal part of the neuroetoderm. Dev. Biol. 189, 186–204.
Scholtz, G., 1992. Cell lineage studies in the crayfish *Cherax destructor* (Crustacea, Decapoda): germ band formation, segmentation and early neurogenesis. Roux's Arch. Dev. Biol. 202, 36–48.
Seugnet, L., Simpson, P., Haenlin, M., 1997. Transcriptional regulation of Notch and Delta: requirements for neuroblast segregation in *Drosophila*. Development 124, 2015–2025.
Simpson, P., 1990. Lateral inhibition and the development of the sensory bristles of the adult peripheral nervous system of *Drosophila*. Development 109, 509–519.
Skeath, J.B., Panganiban, G., Selegue, J., Carroll, S.B., 1992. Gene regulation in two dimensions: the proneural *achaete* and *scute* genes are controlled by combinations of axis patterning genes through a common intergenic control region. Genes Dev. 6, 2606–2619.
Southall, T.D., Brand, A.H., 2009. Neural stem cell transcriptional networks highlight genes essential for nervous system development. EMBO J. 28, 3799–3807.
Stollewerk, A., 2002. Recruitment of cell groups through Delta/Notch signalling during spider neurogenesis. Development 129, 5339–5348.
Stollewerk, A., Weller, M., Tautz, D., 2001. Neurogenesis in the spider *Cupiennius salei*. Development 128, 2673–2688.
Torii, M., Matsuzaki, F., Osumi, N., Kaibuchi, K., Nakamura, S., Casarosa, S., Guillemot, F., Nakafuku, M., 1999. Transcription factors Mash-1 and Prox-1 delineate early steps in differentiation of neural stem cells in the developing central nervous system. Development 126, 443–456.
Ungerer, P., Aspects of Neurogenesis in *Orchestia cavimana* (Amphipoda, Malacostraca, Crustacea). Ph.D. Thesis. Humboldt-University, Berlin, 2006.
Ungerer, P., Eriksson, B.J., Stollewerk, A., 2011. Neurogenesis in the water flea *Daphnia magna* (Crustacea, Branchiopoda) suggests different mechanisms of neuroblast formation in insects and crustaceans. Dev. Biol. 357, 42–52.
Ungerer, P., Scholtz, G., 2008. Filling the gap between identified neuroblasts and neurons in crustaceans adds new support for Tetraconata. Proc. R. Soc. B 275, 369–376.
Weller, M., Tautz, D., 2003. *Prospero* and *Snail* expression during spider neurogenesis. Dev. Genes Evol. 213, 554–566.
Wheeler, S.R., Skeath, J.B., 2005. The identification and expression of *achaete-scute* genes in the branchiopod crustacean *Triops longicaudatus*. Gene Expression Patterns 5, 695–700.
Yasugi, T., Umetsu, D., Murakami, S., Sato, M., Tabata, T., 2008. *Drosophila* optic lobe neuroblasts triggered by a wave of proneural gene expression that is negatively regulated by JAK/STAT. Development 135, 1471–1480.