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Evolution of Developmental Control Mechanisms

Conserved and novel functions for Netrin in the formation of the axonal scaffold and glial sheath cells in spiders

Viktoria Linne, Angelika Stollewerk *

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

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ABSTRACT

Netrins are well known for their function as long-range chemotropic guidance cues, in particular in the ventral midline of vertebrates and invertebrates. Over the past years, publications are accumulating that support an additional short-range function for Netrins in diverse developmental processes such as axonal pathfinding and cell adhesion. We describe here the formation of the axonal scaffold in the spiders *Cupiennius salei* and *Achaearanea tepidariorum* and show that axonal tract formation seems to follow the same sequence as in insects and crustaceans in both species. First, segmental neuropiles are established which then become connected by the longitudinal fascicles. Interestingly, the commissures are established at the same time as the longitudinal tracts despite the large gap between the corresponding hemi-neuromeres which results from the lateral movement of the germband halves during spider embryogenesis. We show that Netrin has a conserved function in the ventral midline in commissural axon guidance. This function is retained by an adaptation of the expression pattern to the specific morphology of the spider embryo. Furthermore, we demonstrate a novel function of *netrin* in the formation of glial sheath cells that has an impact on neural precursor differentiation. Loss of Netrin function leads to the absence of glial sheath cells which in turn results in premature segregation of neural precursors and overexpression of the early motor- and interneuronal marker *islet*. We suggest that Netrin is required in the differentiated sheath cells for establishing and maintaining the interaction between NPGs and sheath cells. This short-range adhesive interaction ensures that the neural precursors maintain their epithelial character and remain attached to the NPGs. Both the conserved and novel functions of Netrin seem to be required for the proper formation of the axonal scaffold.

Introduction

In insects and crustaceans the major axonal tracts of the ventral nerve cord – the transverse commissures and the longitudinal tracts – are established by early differentiating neurons that project along stereotyped pathways (Duman-Scheel and Patel, 1999; Thomas et al., 1984; Whitington, 1995; Whitington and Bacon, 1997; Whitington et al., 1993). These neurons are located in invariant positions in each hemi-neuromere. Comparative analysis of these pioneer neurons in several representatives of insects and crustaceans identified 5 neurons per hemi-neuromere which showed similar cell body positions, time of axonal outgrowth, axonal projections and marker gene expression (Duman-Scheel and Patel, 1999; Goodman and Doe, 1993; Thomas et al., 1984). In the remaining two euarthropod groups, the chelicerates (e.g. spiders) and myriapods (e.g. centipedes), the formation of the axonal scaffold has only been studied in a single representative, the centipede *Ethmostigmus rubripes* (Whitington et al., 1991). In this species the longitudinal tracts are pioneered by neurons that are located in the brain, rather than by segmentally repeated neurons as seen in insects and crustaceans. The formation of the commissures was not analysed and data are missing altogether in chelicerates.

In insects and crustaceans, specialised midline cells are positioned between the bilaterally developing neuropile providing guidance cues for the outgrowing axons (Klämbt et al., 1991; Simanton et al., 2009; Vargas-Vila et al., 2010) Pioneer axons that project ipsilaterally (on the same site of the midline as their cell bodies) are repelled by the midline cells while commissural axons that cross the midline (contralateral projections) are attracted by them. Over the past decade, work in the *Drosophila* central nervous system (CNS) has uncovered a complex system of ligands and receptors that organises axon guidance at the midline. Among them, the Netrins are a family of conserved proteins, structurally related to Laminin, that have retained the function of attracting axons towards the midline from nematodes to vertebrates (Dickson, 2002). The proteins of the Netrin family consist of multiple modules that can be found in functionally divergent proteins. The individual domains were termed VI, V-1, V-2, V-3 and C in the *Caenorhabditis elegans* protein Unc-6 which was the first *netrin* to be identified (Ishii et al., 1992). Netrins can attract different axon populations from short distances of up to a few

* Corresponding author. Fax: +44 20 8983 0973.
E-mail address: a.stollewerk@qmul.ac.uk (A. Stollewerk).

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millimetres, although axons can also be repelled by Netrin and this function is also conserved across the animal phyla (Keleman and Dickson, 2001; Tessier-Lavigne and Goodman, 1996). Two families of receptors mediate the axon guidance function of Netrins: the UNC5 family and the deleted in colorectal cancer (DCC) and neogenin family (reviewed by Cirulli and Yebra, (2007)). Netrin signalling in axonal guidance commonly results in the reorganisation of the actin cytoskeleton preparing cells or cell processes for motility (Shekarabi et al., 2005).

Although Netrin is primarily thought of as a long-range axonal guidance cue, there is increasing evidence for a short-range role of Netrin in cell–cell interactions (Brankatschk and Dickson, 2006; Kennedy, 2000; Manitt et al., 2001; Mawdsley et al., 2004; Srinivasan et al., 2003; Winberg et al., 1998; Yebra et al., 2003). In *Drosophila*, for example, Netrin is required in the ventral neuroectoderm for glial migration (von Hilchen et al., 2010) and in the mammalian CNS Netrin-1 protein is concentrated at the interface between axons and oligodendrocytes indicating a short-range function that mediates neuronal and axon–oligodendroglial interactions (Manitt et al., 2001).

Furthermore, recent findings suggest a role for Netrin in epithelial morphogenesis outside the nervous system (reviewed by Cirulli and Yebra (2007)). This function is mediated by the traditional Netrin receptors as well as receptors of the Integrin family (Baker et al., 2006).

Here we describe the formation of the axonal scaffold in the two spider species *Cupiennius salei* and *Achaearanea tepidariorum* and analyse the expression pattern of *netrin* during this process. Furthermore, we analyse the function of Netrin in *C. salei* by RNA interference.

The following paragraph gives a brief summary of spider embryogenesis to familiarise the reader with the anatomy of the relevant stages.

The early embryonic development of *A. tepidariorum* and the complete development of *C. salei* (chelicerates) have been described in detail by Akiyama-Oda and Oda, (2003) and Seitz, (1966), respectively. Both spider species exhibit essentially the same mode of development, although embryogenesis in *C. salei* takes twice as long as in *A. tepidariorum* (unpublished results; see Materials and methods). After formation of the germband, the 7 prosomal segments (head and thorax) form simultaneously while the 11 opisthosomal segments (abdomen) are added sequentially. Due to this mode of segment formation, the development of the prosoma is more advanced throughout embryogenesis as compared to the opisthosomal segments. When the germband reaches its longest extension before mid-embryogenesis a process called inversion is initiated (Anderson, 1973). During this process the spider embryo undergoes major morphological changes transforming from an elongated germband into the typical spider shape. Inversion involves the splitting of the germband into left and right halves (Suppl. Fig. 1). The two halves separate along the ventral midline and move towards lateral and dorsal. The extending gap between them is covered by a single layered epithelium which was termed ventral sulcus (Anderson, 1973; Seitz, 1966). We will refer to this structure here as ventral midline. The ventral midline epithelium continuously expands while the germband halves migrate laterally (Suppl. Fig. 1). It reaches its widest expansion when the embryo has closed dorsally and is beginning to bend around a transverse furrow between prosoma and opisthosoma at about 250 h of development in *C. salei* and 120 h in *A. tepidariorum*. After dorsal closure, when both germband halves move towards each other again, the ventral midline epithelium becomes reduces and dissolves at ventral closure at the end of embryogenesis at around 350 h (*C. salei*) and 180 h (*A. tepidariorum*), respectively (Suppl. Fig. 1). Embryogenesis is followed by postembryonic development in which the spiders moult several times until the mature adult emerges (Foelix, 1996).

The splitting of the germband halves and the particular morphogenetic movements of the spider embryo occur during the formation of the nervous system. Both in chelicerates and in myriapods the nervous system is generated by groups of neural precursors (NPGs) (Chipman and Stollewerk, 2006; Dove and Stollewerk, 2003; Kadner and Stollewerk, 2004; Mittmann, 2002; Pioro and Stollewerk, 2006; Stollewerk et al., 2001). These groups appear in several phases at stereotyped positions in the neuroectoderm. We have shown recently that the pattern of NPGs is almost identical in the spiders *C. salei* and *A. tepidariorum* and largely conserved in myriapods (Döffinger and Stollewerk, 2010). The neural precursors segregate from the groups and most of them directly differentiate into neurons or glial cells. In contrast, in insects and crustaceans single stem cell-like neuroblasts are selected that divide asymmetrically and produce neural precursors (Goodman and Doe, 1993; Ungerer and Scholtz, 2008). These divide again to generate the neurons and/or glial cells that form the characteristic ladder-like axonal scaffold of arthropods. Both in insects and crustaceans, the ventral midline is only a few cells wide and consists of specialised cells that provide guidance cues for commissural axons among others (Klämbt et al., 1991; Simanton et al., 2009). This raises the question how the axonal scaffold in spiders forms, notably the commissures – given the particular morphogenetic movements of the spider germband – and in what ways conserved guidance molecules are involved in this process.

We show here that Netrin has a conserved function in the commissural axon guidance in both spider species. Furthermore, we demonstrate a novel role for Netrin in glial sheath cells that enwrap the neural precursor groups in the ventral neuromeres in *C. salei*. Both functions seem to be required for the proper formation of the axonal scaffold.

Materials and methods

Spider stocks and developmental stages

Fertilised females of the Central American wandering spider *C. salei* Keyserling were obtained from Ernst-August Seyfarth, University of Frankfurt, Germany. Embryos were collected as described before (Stollewerk et al., 2001). *A. tepidariorum* males and females for breeding were kindly provided by Hiroki Oda, University of Osaka, Japan and Alistair McGregor, Veterinärmedizinische Universität Wien, Austria. Spiders were cultured and embryos were collected as described by (Akiyama-Oda and Oda, 2003). The embryonic stages of *C. salei* were classified according to Seitz (1966). The embryonic development of *A. tepidariorum* takes about half as long as that of *C. salei* (unpublished results). The table shows the developmental times for corresponding stages in *A. tepidariorum* and *C. salei*.

C. salei	150 h	170 h	200 h	230 h	280 h	300 h
A. tepidariorum	75 h	90 h	100 h	115 h	140 h	150 h

PCR cloning

Cs netrin was identified by RT-PCR on cDNA obtained from RNA of 180 h embryos using degenerate primers directed against the conserved *netrin* domains VI and V-3. The following primers were used for *C. salei*: GNM GNT GYA THC CNG AYT TYG (forward); TGY TTR CAN GGR CAY TGN CC (reverse). PCR fragments of 1140 bp length were amplified, cloned and sequenced. The following primers were used to amplify a 640 bp fragment from *A. tepidariorum* cDNA: GNM GNT GYA THC CNG AYT TYG (forward) and GCR TGN CCR TTR CAY TTR CA (reverse). The sequences were deposited in the GenBank database. Accession numbers: JF302896 (*Cs netrin*); JF302897 (*At netrin*).

Histology and staining

Whole-mount in situ hybridisations and immunocytochemistry were performed as described previously (Akiyama-Oda and Oda, 2003; Stollewerk et al., 2001) Phallodin-FITC was purchased from