Comparative analysis of midline guidance and axon pathfinding in the chelicerates *Achaearanea tepidariorum* and *Cupiennius salei*

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Zusammenfassung

Die Neurogenese und axonale Wegfindung sind in den vergangenen Jahrzehnten Thema einer Vielzahl wissenschaftlicher Untersuchungen in den verschiedensten Organismen gewesen. Die zusammengetragenen Daten in Insekten und Crustaceen geben eine gute Übersicht darüber, wie das Nervensystem in Arthropoden aufgebaut wird. Die entwicklungsbiologischen Prozesse, die daran beteiligt sind, sind in den beiden genannten Gruppen sehr gut verstanden. In den Gruppen der Cheliceraten und Myriapoden jedoch wurden ähnliche Analysen bisher kaum durchgeführt. Das Hauptanliegen dieser Arbeit war es daher, Mechanismen in den Spinnen *Achaearanea tepidariorum* und *Cupiennius salei*, zwei Vertretern der Cheliceraten, zu untersuchen, die eine Rolle im Leitsystem der ventralen Mittellinie und bei der axonalen Wegfindung spielen. Eine Vorraussetzung hierfür sind Kenntnisse über die Architektur des Zentralnervensystems. In einem ersten Schritt beschrieb ich daher grundlegend die Morphologie des Nervensystems im Verlauf der gesamten Embryoalentwicklung. Ich konnte zeigen, dass in Spinnen ein für Arthropoden typisches Strickleiternervensystem gebildet wird. Dieses wird von segmental angelegten Neuronen geformt, wobei sowohl Gruppen von Zellen als auch einzelne Neurone daran beteiligt sind, die primären axonalen Trakte zu etablieren. Im Besonderen konnte ich eine Zelle identifizieren, die in Position, Projektionsmuster und der Expression des Markergens *even-skipped* vergleichbar zum PR2 Neuron in *Drosophila* ist, welches die posteriore Wurzel des Segmentalnervs anlegt.

In einem zweiten Ansatz untersuchte ich die ventrale Mittellinie in Spinnen im Bezug auf ihre mögliche Funktion in der axonalen Wegfindung. Es konnte gezeigt werden, dass es sich beim Epithel der Mittellinie, das die Lücke zwischen beiden Keimstreifhälften während des gesamten Prozesses der Inversion überspannt, um eine transiente Struktur handelt, die keine neuralen Zellen hervorbringt. Es ist daher vergleichbar mit der so genannten Floor plate in Vertebraten, die ebenfalls nur vorübergehend existiert. Die Untersuchung von *single minded* (*sim*) zeigte, dass es, anders als in *Drosophila*, wo *sim* ein wichtiges regulatorisches Gen für die korrekte Spezifizierung von Mittellinienzellen ist, nicht in den Zellen der Mittellinie, sondern in diesen benachbarten Zellen, exprimiert wird. Das ist vergleichbar mit Vertebraten.

Zusätzlich konnte ich Expression von *sim* an den Basen der Gliedmassen und im Kopf nachweisen. Wie in Vertebraten könnte *sim* an der Musterbildung dieser Gewebe beteiligt sein. Dennoch spielt die Mittellinie in Spinnen eine wichtige Rolle als Organisator für auswachsende, kommissurale Axone. Diese Funktion teilt sie mit anderen Invertebraten und Vertebraten.

Die Signaltransduktionskaskade, die an der axonalen Wegfindung an der Mittellinie beteiligt ist, ist in den verschiedensten Organismen hoch konserviert. In der vorliegenden Arbeit konnte ich sowohl in *Achaearanea* als auch in *Cupiennius* ein *netrin* Homolog identifizieren und eine konservierte Funktion des Wegfindungsmoleküls während der Bildung der Kommissuren aufzeigen. RNAi Experimente belegen, dass, wird die Funktion von *netrin* herunterreguliert, das Strickleiternervensystem nicht korrekt gebildet wird, ins Besondere die kommissuralen Faszikel. Des Weiteren konnte ich eine neue Funktion von *netrin*, die bisher in anderen Organsimen noch nicht beschrieben wurde, identifizieren. Neben seiner Rolle in der axonalen Wegfindung, scheint *netrin* auch an der epithelialen Morphogenese im zentralen Nervensystem beteiligt zu sein. In dieser Funktion scheint *netrin* in Gliazellen, die die epithelialen Vesikel der Invaginationsgruppen umhüllen, wichtig zu sein, um neurale Vorläuferzellen in einem undifferenzierten Zustand zu halten. Der Abbau von *netrin* Transkript durch RNA Interferenz führt zu einer verfrühten Segregation neuraler Vorläuferzellen aus dem epithelialen Verband der Invaginationsgruppen und zu einer Zunahme an Zellen, die den frühen Differenzierungsmarker *islet* exprimieren.

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1 Introduction

Arthropoda, by far the largest and most diverse animal taxon, including chelicerates, crustaceans, insects and myriapods, have conquered all biological habitats. Correspondingly the arthropod central nervous system (CNS) shows a high variation of neural structures adapted to the specific requirements of behaviour and lifestyles of the individual species. These adaptations must be the result of evolutionary modifications in the developmental processes involved in generating the nervous system. Comparative analysis of arthropod CNS development will improve our understanding of the principles of neural development, the phylogeny of arthropods and the way in which evolutionary changes in development might have produced neural diversity.

1.1 Nervous system development in arthropods

Despite the huge variety of nervous systems in the arthropod phylum, the basic organisation is rather similar in all four groups. The arthropod nervous system comprises of an anterior-dorsal brain and the ventral nerve cord (VNC), an assembly of segmentally organised neuromeres, which are interconnected by transverse nerve tracts within one segment (commissures) and longitudinal fascicles between consecutive segments (connectives) to form a rope ladder-like structure (Paulus and Weygold, 1996). Within arthropods our knowledge of neurogenesis is largely restricted to insects, namely to the fruit fly *Drosophila melanogaster* (*Drosophila*), which has been the subject of intense research over the last decades (Doe and Goodman, 1985a; Hartenstein and Campos-Ortega, 1984). In crustaceans, the probably most closely related sister group to insects (Giribet et al., 2001; Mallatt et al., 2004; Peterson and Eernisse, 2001; Pisani et al., 2004; Regier and Shultz, 2001; Regier et al., 2010; Shultz and Regier, 2000), data on neurogenesis are still relatively sparse. In chelicerates and myriapods even less is known and understanding of neurogenic processes is only beginning to emerge (for an overview see Stollewerk and Simpson, 2005). The following paragraph will give an overview of the

mechanisms involved in neurogenesis in arthropods to emphasise similarities and differences.

The neural cells of the VNC in arthropods stem from progenitor cells, which are recruited from the neuroectoderm (NE). The competence to take on a neural cell fate depends on the activity of the proneural genes of the *acheate-scute* complex, which have been identified throughout the insect phylum (Broadus and Doe, 1995; Cabrera et al., 1987; Galant et al., 1998; Jimenez and Campos-Ortega, 1990; Martin-Bermudo et al., 1991; Pistillo et al., 2002; Romani et al., 1989; Skaer et al., 2002; Skeath and Carroll, 1992; Wheeler et al., 2003; Wulbeck and Simpson, 2002). Through mediation of the neurogenic genes *Notch* and *Delta*, in *Drosophila* a single cell is selected from the proneural clusters to become a neurogenic stem cell, the so-called neuroblast (NB) (Lehmann et al., 1981; Lehmann et al., 1983; Campos-Ortega and Hartenstein, 1997; Bourouis et al., 1989). Eventually in insects about 30 NBs are generated per hemisegment, which delaminate sequentially and are arranged in a stereotypic pattern (Bate, 1976b; Broadus and Doe, 1995; Doe and Goodman, 1985a; Jimenez and Campos-Ortega, 1990; Skeath et al., 1994; Wheeler et al., 2003; Wheeler et al., 2005). An individual cell fate is assigned to each NB defined by the position from which it delaminates and the time of its birth, resulting in a unique combination of genes being expressed (Bhat, 1996; Doe, 1992; Isshiki et al., 2001; Skeath, 1999; Skeath and Carroll, 1994; Skeath and Doe, 1996; Truman and Bate, 1988). After delamination *Drosophila* NBs start to divide asymmetrically in a stem cell mode to renew the NB and give rise to a ganglion mother cell (GMC), which divides once thus generating a specific invariant cell lineage consisting of neurons and/or glial cells (Bossing et al., 1996b; Broadus et al., 1995; Doe, 1992; Goodman and Doe, 1993; Skeath, 1999).

In the crustaceans that have been analysed also single NBs are generated in the NE (Dohle, 1976; Scholtz, 1990; Gerberding, 1997; Harzsch, 2001). In higher crustaceans the NB can produce not only neural cells but also epidermal cells after the generation of GMCs (Dohle, 1976; Scholtz, 1990; Doe and Goodman, 1985a; Harzsch, 2001; Scholtz, 1992; Gerberding, 1997; Dohle and Scholz, 1988; Ungerer and Scholtz, 2008).

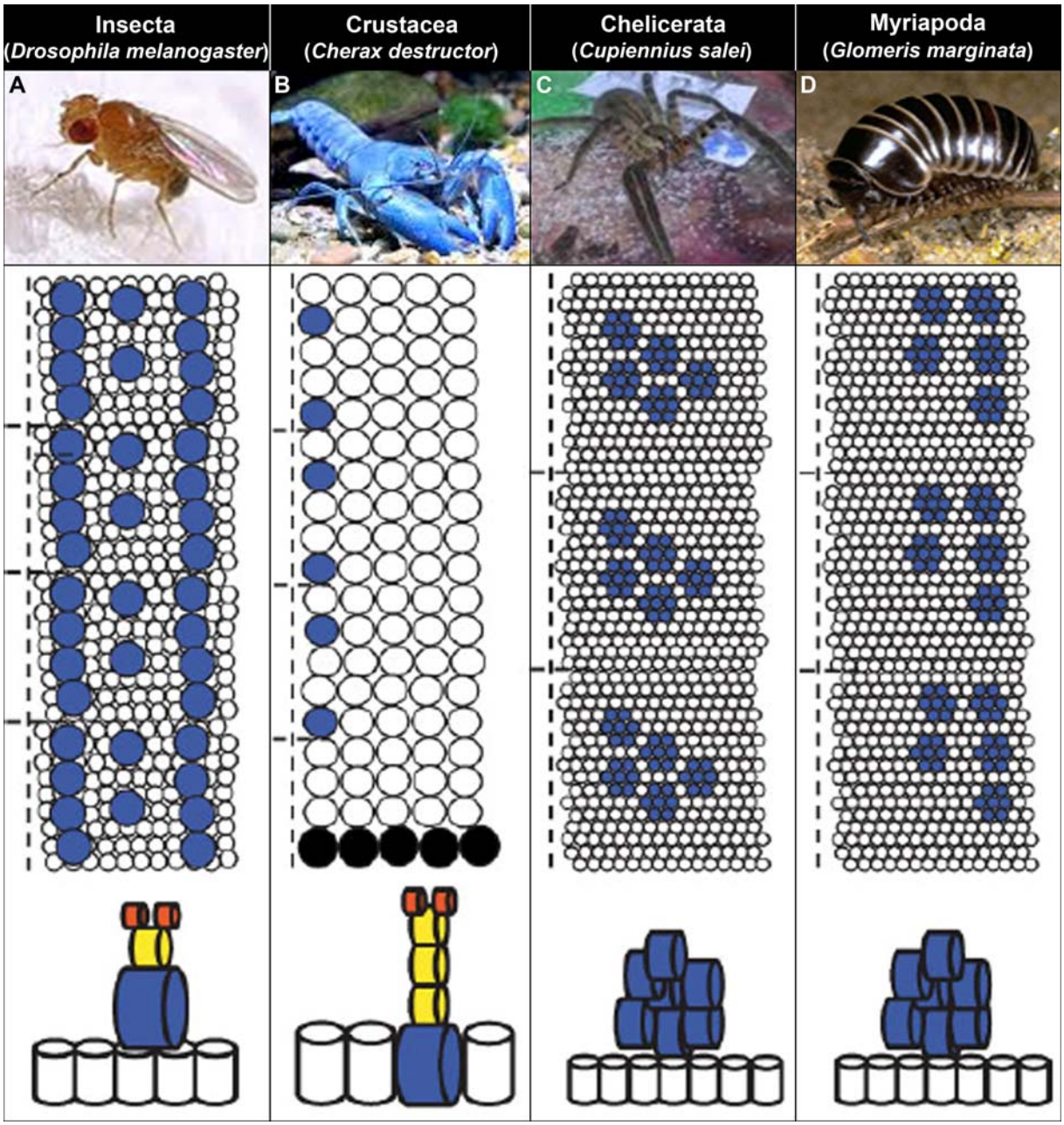

Figure 1-1: Schemata of neural precursor formation in arthropods.

Within the four arthropod groups different modes of neural precursor formation have evolved. The drawing depicts precursor formation in four hemineuromeres in insects (**A**), and crustaceans (**B**) and in three hemisegments in chelicerates (**C**) as well as in myriapods (**D**). (**A**) In insects about 30 neural stem cells (NBs) are recruited from the NE in five distinct waves (shown in blue are NBs of the first round of delamination). After delamination the NB (blue) divides in a stem cell mode to renew itself and give rise to a ganglion mother cell (yellow, GMC), which in turn divides once to generate neurons and/ or glia cells (red). (**B**) In crustaceans individual neural stem cells (NBs, blue) are generated in the apical layer and divide in a similar mode as the insect NBs. They divide asymmetrically to produce GMCs (yellow), which then generate neuronal and/ or glial progeny (red). Differently from insects, NBs in the crustaceans analysed do not delaminate but remain in the apical layer. (**C**, **D**) In chelicerates and myriapods instead of single precursor cells whole groups of cells adopt the neural cell fate (blue) and invaginate from the apical layers of the NE. These precursor cells are no stem cells as the insect and crustacean NBs and do not divide any further. Dashed lines mark the midline and the segmental boundaries. Modified after Stollewerk and Simpson, 2005.

The recruitment of neural precursors in chelicerates and myriapods relies on homologues of the proneural and neurogenic genes, similar to *Drosophila* (Dove and Stollewerk, 2003; Kadner and Stollewerk, 2004; Mittmann, 2002; Stollewerk, 2002; Stollewerk et al., 2001). However, different to insects and crustaceans not single NBs are selected but whole groups of cells (neural precursor groups; NPGs) adopt the neural fate. Another difference is that in chelicerates and myriapods no asymmetric cell divisions could be observed in the apical layers supporting the idea that no individual neural stem cells are present (Anderson, 1973; Stollewerk et al., 2001; Weller and Tautz, 2003). Nevertheless, in each hemisegment about 30 NPGs are generated sequentially and are arranged in a stereotypic pattern (Döffinger and Stollewerk, 2010; Dove and Stollewerk, 2003; Kadner and Stollewerk, 2004; Stollewerk et al., 2001).

In insects it was shown that neural stem cells generated during early neurogenesis can be stored intermediately before entering the cell cycle again to generate neurons that accommodate the need for changes during the life cycle and to integrate newly formed structures into the existing neuronal network. Such postembryonic NBs (pNBs), after a phase of quiescence, resume asymmetric divisions similar to embryonic NBs (Booker and Truman, 1987; Ito and Hotta, 1992; Prokop and Technau, 1991; Truman and Ball, 1998; Truman and Bate, 1988). Similar findings are reported from crustacean larvae (Harzsch and Dawirs, 1996; Harzsch et al., 1998; Schmidt, 1997). Similarly, in myriapods and chelicerates, during larval stages new neural structures are formed that need to be incorporated into the existent nervous system (Anderson, 1973; Gold et al., 2009; Seyfarth et al., 1995; Stollewerk and Seyfarth, 2008). It has been shown recently in *Cupiennius* that additionally to the primary neural precursor groups (pNPGs), secondary neural precursor groups (sNPGs) arise towards the end of embryonic development that do not all differentiate until larval stages. Differently from the primary groups, the sNPGs do not detach from each other after invagination, instead they maintain the epithelial character and form so-called epithelial vesicles. About half of the cell groups are still detectable in larval stages, providing neural progenitors for larval and possibly adult neural structures (Stollewerk, 2004).

1.2 Axon pathfinding

However diverse adult nervous systems in arthropods might be, the above described mechanisms indicate that the basic construction of the early nervous system might be conserved in arthropods, despite all differences. In a similar way later neurodevelopmental events, such as the establishment of the embryonic axon scaffold could be conserved.

1.2.1 Axonogenesis in arthropods

In insects, that have been subject to elaborate analysis concerning axon pathfinding, the central and peripheral axonal pathways, which connect the individual neuromeres and form the typical arthropod nervous system structure are established by segmentally organised neurons (Boyan et al., 1995; Goodman and Doe, 1993; Thomas et al., 1984). These so-called pioneer neurons differentiate from early delaminating NBs. Specialised midline cells provide positional information and guidance cues for the outgrowing axons (Harris et al., 1996; Klämbt and Goodman, 1991; Klämbt et al., 1991; Mitchell et al., 1996; Tear et al., 1996). Early developing axons, originating from the pioneer neurons, take stereotypic routes to establish the primary axonal tracts. Those that project ipsilaterally (on the same side of the midline as their cell bodies) are repelled by the midline cells, while commissural axons are attracted towards the ventral midline. After midline crossing commissural axons must be deterred from re-crossing. The growth cones of later developing neurons can track the pathways primed by the pioneering cells to reach their correct targets (Goodman et al., 1984; Thomas et al., 1984; Whitington, 1993).

Comparative studies on early axonogenesis in insects and crustaceans (Duman-Scheel and Patel, 1999; Thomas et al., 1984; Whitington et al., 1993a; Whitington, 1995) have uncovered a set of five pioneer neurons, which can be homologised in the two arthropod groups. Criteria for homology were the position of the cell bodies, similarity in axonal projections and the expression of similar molecular markers. However, data on axonal guidance and the establishment of the embryonic axon scaffold in chelicerates and myriapods are basically not existent. Merely in the

centipede *Ethmostigmus rubripes* an investigation on the establishment of axonal tracts had been conducted. Here the earliest central axon pathways, which are established along the anterior-posterior axis, do not arise from segmentally repeated neurons as was shown in insects but by the posterior growth of axons originating from neurons located in the brain (Whitington et al., 1991). In addition, the axonal projections and cell body positions of segmental neurons clearly diverge from the pattern described in insects and crustaceans (Whitington et al., 1991; Whitington, 1995). In chelicerates the cell soma positions and axonal projections of several neurons immunoreactive for neurotransmitter have been analysed. No clear overlaps could be found with the insect and crustacean pattern (Agricola and Braunig, 1995; Wegerhoff and Breitbach, 1995; Breidbach et al., 1995). In chelicerates no analysis has been conducted on the establishment of the axon scaffold to date.

1.2.2 Axon pathfinding in vertebrates

Axonal patterning of the vertebrate spinal cord shares several features with the mechanisms known from insects. In lower vertebrates early axon pathways are pioneered by a number of early neurons that are characteristic in position. Following the vertebrate terminology these are termed primary neurons compared to pioneer neurons in invertebrates (Chitnis and Kuwada, 1990; Forehand and Farel, 1982; Roberts and Clarke, 1982; Wilson et al., 1990). The trajectories of these neurons follow specific, stereotypic routes. Commissural neurons are located at dorso-lateral positions on either side of the spinal cord and extend axons along a circumferential path towards the floor plate. Pioneer axons travel along the lateral edges of the spinal cord until they reach the floor plate, the follower axons initially take the same route but eventually turn away from the lateral edges and then pursue a more ventro-medial route (reviewed by Colamarino and Tessier-Lavigne, 1995). After reaching the midline, commissural axons cross through the ventral-most third of the floor plate (Bovolenta and Dodd, 1990). Another subset of early developing neurons located in similar positions as the commissure pioneering neurons initially choose the same route towards the floor plate but instead of crossing they extend parallel to the midline and project ipsilateral to pioneer the longitudinal tracts (Colamarino and Tessier-Lavigne, 1995).

Comparisons of insect and vertebrate data suggest that the primary neurons in both are phylogenetically old (Korzh et al., 1993; Thomas et al., 1984; Whitington, 1995). They send out pioneer axons that establish an early axon scaffold for continued axonal outgrowth in a similar fashion. For many developing axons, in vertebrates and invertebrates the midline functions as landmark on their way to reach their appropriate targets and thus, given their position and early differentiation, serve as equivalent structures.

1.3 The ventral midline is an important organising centre in nervous system development

In bilaterial organisms the ventral midline defines the axis of symmetry between the two halves of the NE and is a critical organising centre for the developing CNS. The specialised population of midline cells are among the first cells to differentiate during embryonic development (Crews et al., 1988; Schoenwolf and Smith, 1990; Thomas et al., 1988). Although the specification of midline cell populations differs (Ang and Rossant, 1994; Crews et al., 1988; Klämbt et al., 1991; Menne and Klambt, 1994; Nambu et al., 1990; Weinstein et al., 1994), the *Drosophila* mesectoderm and the vertebrate notochord and floor plate are thought to represent common elements of a conserved body plan that is similar in vertebrates and invertebrates (Arendt and Nubler-Jung, 1996). Midline cells have in common to represent inductive centres for the regional patterning of the CNS and other organ tissues. Furthermore insect and vertebrate midline cells play a major role in the establishment of axonal tracts since they are the source of guidance molecules, which are involved in axon pathfinding (Briscoe and Ericson, 1999; Briscoe and Ericson, 2001; Ericson et al., 1996; Golembo et al., 1996; Kim and Crews, 1993; Lewis and Crews, 1994; Luer et al., 1997; Menne et al., 1997; Placzek et al., 1991; Sasai and De Robertis, 1997; Schweitzer et al., 1995; Yamada et al., 1991).

In *Drosophila* the ventral midline derives from the mesectoderm, a narrow epithelial stripe positioned in-between the mesoderm and the NE. During gastrulation the mesoderm invaginates and the mesectodermal stripes of both embryonic halves come to lie next to each other (Campos-Ortega and Hartenstein, 1997), whereupon

the midline cells, that initially maintain adhesive contacts with the mesoderm, loose their adherent properties and are internalised. Even before the onset of gastrulation the midline precursor cells themselves are distinguishable by morphological and molecular criteria (Crews et al., 1988; Poulson, 1950). From the midline progenitors arise a variety of different neurons and glial cells including the midline glia (MGP, MGM, MGA) and several neurons (MP1, VUM, MNB) comprising a set of about 20 individual cells per segment (Bossing and Technau, 1994; Jacobs and Goodman, 1989a; Klämbt et al., 1991). These cells play an important role in the formation of commissures and longitudinal tracts as they provide guidance molecules for outgrowing axons (Harris et al., 1996; Klämbt et al., 1991; Mitchell et al., 1996; Tear et al., 1996).

In vertebrates the development of the nervous system begins with the induction of the neural plate from undifferentiated ectoderm in response to inductive signals that originate in the adjacent mesoderm (Dixon and Kintner, 1989; Jacobson, 1988; Placzek et al., 1990; Ruiz i Altaba, 1994; Savage and Phillips, 1989; van Straaten et al., 1989). Comparable events might control the development of the *Drosophila* ventral midline as well (Kosman et al., 1991; Leptin, 1991). Through folding and invagination the neural tube is formed, its ventral-most part is specified as the ventral midline or floor plate (Kingsbury, 1930). Like in *Drosophila*, the cells of the vertebrate midline are the first cells to differentiate (Bancroft and Bellairs, 1975; Jessell et al., 1989; Schoenwolf, 1982). Morphologically the floor plate is made up of characteristic columnar cells that occupy the neural tube at its ventral midline and comprise distinct populations of glial cells (McKanna, 1993). In contrast to the fly, the vertebrate floor plate does not give rise to any neurons and only exists as a transient structure during embryonic development. Nevertheless, the cells of the floor plate function as a key landmark and organising centre and appear to provide guidance information for ventrally navigating axon growth cones in the embryonic spinal cord, playing important roles in organising both longitudinal projections near the midline and some circumferential projections including the commissures (reviewed by Colamarino and Tessier-Lavigne, 1995). The floor plate also controls neuronal differentiation and may have a role in the determination of cell identity and patterning at earlier stages of neural tube development (Colamarino and Tessier-Lavigne, 1995; Pourquié et al., 1996).

1.4 *Single minded* is the key regulator of midline cell fate in *Drosophila* and important in CNS development in vertebrates

In *Drosophila*, *single minded* (*sim*) is the master regulatory gene controlling midline cell fate determination. *Sim* mutants are characterised by the loss of midline precursor cells, which results in the improper formation of the entire embryonic axon scaffold. Commissural tracts in each segment are largely missing and the longitudinal axon tracts appear to have fused ventrally (Thomas et al., 1988). Furthermore, *sim* is required for the transcription of a set of midline genes (*slit*, *Toll*, *rhomboid* and *engrailed*), all of which were shown to be involved in the differentiation of midline progenitors and thus participate in axon guidance (Nambu et al., 1990). The first evidence of *sim* transcript is at the blastoderm stage in cells of the mesectoderm. During gastrulation the ventral midline is formed, the midline cells differentiate and *sim* is expressed in all midline cells along the anterior-posterior axis. Expression of *sim* is maintained throughout embryonic development, although later it becomes restricted to the midline glia (Crews et al., 1988; Nambu et al., 1991; Thomas et al., 1988). Additionally to the ventral nervous system *sim* was found to be expressed in a number of muscle precursor cells, the gut and the brain, where it might function in regionalisation and cell fate determination (Crews et al., 1988; Lewis and Crews, 1994).

In vertebrates, homologues of *Drosophila sim* where identified in different species. One or two genes were found in mice (Ema et al., 1996; Fan et al., 1996), in chick (Pourquié et al., 1996), zebrafish (Serluca and Fishman, 2001; Wen et al., 2002) and in Xenopus (Coumailleau et al., 2000). The vertebrate *sim* genes are expressed in a variety of different tissues, neural and nonneural, such as the brain, ventral spinal cord, in the developing kidney and in muscles reminiscent of the distribution in *Drosophila*. Importantly, whereas *sim* is essential as key regulator of CNS midline cell development in the fly, the mammalian *Sim* genes are not expressed in floor plate cells of the spinal cord but in a subset of cells immediately adjacent to midline cells (Ema et al., 1996; Fan et al., 1996). In chick *sim* expressing cell populations were identified positioned between motor neurons and the floor plate (Pourquié et al., 1996). Nevertheless, also the vertebrate *Sim* genes play an important role in CNS

development (Eaton and Glasgow, 2006; Goshu et al., 2002; Michaud et al., 1998; Shamblott et al., 2002).

Sim has also been identified in other insects (Zinzen et al., 2006), where it was shown to be expressed in a similar fashion as in *Drosophila*. However, not much data exist for the other arthropod groups. Only in the spider *Achaearanea tepidariorum* (*Achaearanea*) *sim* has been cloned recently and is expressed during early development when the germ band is fully extended in a single ventral stripe along the anterior-posterior axis of the embryo. However, these cells could not be distinguished morphologically from the neighbouring NE (Akiyama-Oda and Oda, 2006).

1.5 *Netrin* functions in midline guidance in *Drosophila* and in vertebrates

Work on the *Drosophila* nervous system and in vertebrates has uncovered a complex system of ligands and receptors, which controls axon pathfinding at the ventral midline (reviewed by Dickson, 2002; Kaprielian et al., 2001). Many of these factors are highly conserved across species and so are the Netrins, which as secreted ligands form a concentration gradient to be interpreted by growth cones (reviewed by Barallobre et al., 2005; Livesey, 1999). To date a single *netrin* has been described in several invertebrates such as *Artemia franciscana*, *C. elegans* and *Branchiostoma floridae* (Duman-Scheel et al., 2007; Hedgecock et al., 1990; Ishii et al., 1992; Shimeld, 2000; Simanton et al., 2009). In *Drosophila*, two Netrins exist, which are expressed in cells of the ventral midline where they direct commissural and longitudinal axon growth (Harris et al., 1996; Mitchell et al., 1996). Vertebrates, where Netrins were first discovered, show a greater diversity concerning the number of orthologues that fulfil numerous functions (de la Torre et al., 1997; Kennedy et al., 1994; Koch et al., 2000; Lauderdale et al., 1997; Lin et al., 2003; Meyerhardt et al., 1999; Nakashiba et al., 2002; Serafini et al., 1996; Serafini et al., 1994; Strahle et al., 1997; Wang et al., 1999). Floor plate cells secrete the morphogen to attract and guide axons to cross the midline during axon pathfinding (Kennedy et al., 1994; Placzek et al., 1990; Tessier-Lavigne et al., 1988).

Inactivation of *netrin* leads to severe pathfinding defects. In vertebrates most commissural axons can no longer extend to reach the floor plate and thus axonal trajectories across the midline are severely affected (Serafini et al., 1996). In the fly, in *netrin* double mutants, commissural projections are thinner than in wildtype, indicating that fewer fibres than normal have crossed the midline. Genetic analysis revealed that both genes play redundant roles in the midline (Harris et al., 1996; Mitchell et al., 1996). Netrins have been shown to act as long range guidance cues as well as at short distances (Brankatschk and Dickson, 2006; Kennedy et al., 1994; Placzek et al., 1990; Serafini et al., 1994). Dependent on the receptor they bind to, Netrins can also either convey an attractive impulse (Chan et al., 1996; Keino-Masu et al., 1996; Kolodziej et al., 1996) or give repulsive cues (Keleman and Dickson, 2001; Labrador et al., 2005; Leonardo et al., 1997; Leung-Hagesteijn et al., 1992). In addition to providing guidance cues for axons, recent data hint at other functions of *netrin* in nervous system development as well as developmental processes beyond the CNS. Studies in vertebrates on pancreas, lung and mammary gland suggest that Netrins provide positional information for cell movement, proliferation and cell survival. Furthermore, Netrins are thought to influence epithelial and vascular morphogenesis (summarised in Cirulli and Yebra, 2007; Kennedy, 2000). Cell-cell interactions involving Netrin signalling were also postulated in *Drosophila*, where *netrin* was shown to be expressed at nerve-muscle synapses (Winberg et al., 1998).

1.6 Anatomy of the adult nervous system in spiders

Although little is known about the formation of the embryonic axon scaffold in spiders or chelicerates in general, the adult nervous system has been described in some detail. In the adult spider the CNS comprises the supraoesophageal ganglion, which is the conglomerate of the cheliceral ganglia fused with the protecerebrum (Hanström, 1926; Weltzien, 1988; Weltzien and Barth, 1991; Babu and Barth, 1984; Babu, 1985; Doeffinger et al., 2010). The second unit of the CNS, the subesophageal ganglion is formed by the neuromeres of the paired pedipalps, four pairs of walking legs and the condensed abdominal nerve mass (Fig. 1-2, Foelix, 1996). Longitudinal and commissural tracts, which show repetitive organisation, connect the condensed neuropile. In *Cupiennius* the suboesophageal ganglion complex comprises of 11

pairs of longitudinal tracts and five commissures that are distributed in dorso-ventral direction. The periphery is innervated by laterally outgrowing projections. In the pedipalp and leg ganglia two neuropiles, a dorsal motoric and ventral sensoric, were identified (Babu, 1965; Babu and Barth, 1984, 1985, 1989, Barth, 1985; Wegerhoff and Breitbach, 1989; Breidbach and Wegerhoff, 1993).

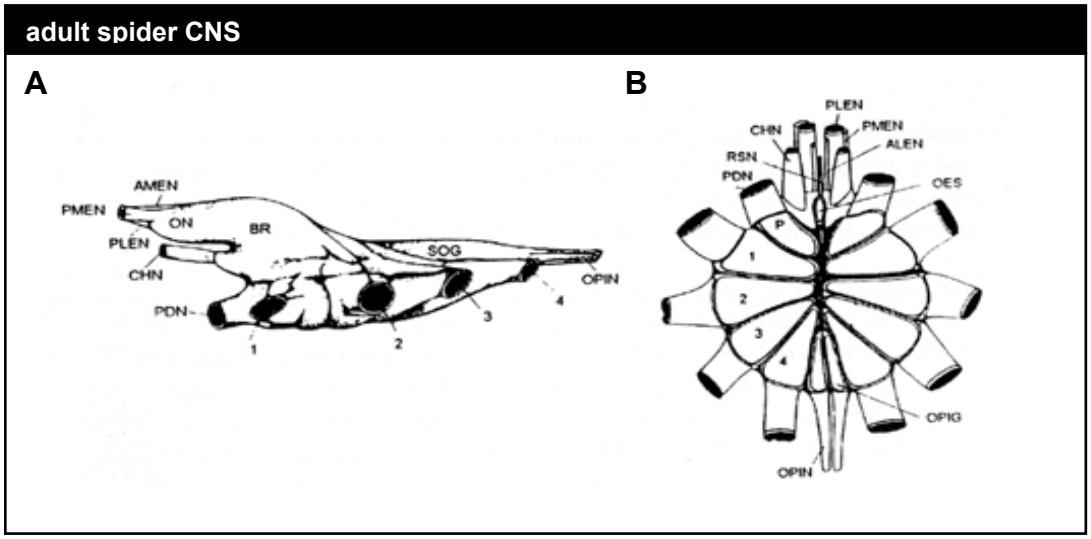

Figure 1-2: Morphology of the adult CNS of *Cupiennius salei*.

The CNS in *Cupiennius salei* comprises of the subraoesophageal and the suboesophageal ganglion. The first is formed by the cheliceral neuromeres fused with the brain and the latter consists of the neuromeres of the pedipalps, leg segments and the condensed abdominal nerve mass. (**A**) Lateral view. (**B**) Ventral view. **1**-**4**; leg ganglia 1-4, **ALEN**; anterior lateral eye nerve, **AMEN**; anterior median eye nerve, **BR**; brain, **CHN**; cheliceral nerve, **OES**; oesophagus, **OPIG**; opisthosomal ganglion, **OPIN**; opisthosomal nerve, **ON**; optic neuropile, **P**; pedipalpal ganglia, **PDN**; pedipalpal nerve, **PLEN**; posterior lateral eye nerve, **PMEN**; posterior median eye nerve, **RSN**; rostral nerve, modified after Wegerhoff and Breidbach, 1995.

1.7 Embryonic development of spiders

The early embryonic development in spiders has been subject of several studies in recent years (McGregor et al., 2008) and been described in-depth for *Cupiennius salei* by Seitz (1966) and *Achaearanea tepidariorum* by Akiyama-Oda and co-workers (2003). In both spider species development is very similar, albeit in *Cupiennius* it takes about twice as long as in *Achaearanea*. In the early embryo the body axes are set up, the germ band develops and the prosomal segments form. The segmented germ band elongates by sequential addition of opisthosomal segments in an anterior-posterior fashion, while the prosomal limb buds appear. Appendages of the prosoma comprise the pedipalps, the chelicerae and four pairs of walking legs.

Eventually, the last abdominal segments are added, the opisthosoma consists of 11 segments and the telson (Anderson, 1973). At the same time, limb buds are formed in opisthosomal segments o2-o5, that give rise to the book lungs, trachea and the spinnerets. The first abdominal segment bears no appendages and will be reduced to become part of the separation between prosoma and opisthosoma. In *Cupiennius* this process takes about 200 hours (h) after egg laying (AEL) at 25°C and around 100h in *Achaearanea* (*Cupiennius* stages after Seitz, 1966; stages of *Achaearanea*, personal communication Dr Beate Mittmann). When the germ band reaches its full length and the anterior and posterior most tips of the embryo almost touch each other (180-190h in *Cupiennius*) a process called inversion is initiated (Fig. 1-3). During this process the spider embryo undergoes tremendous morphological changes, in which the embryo transforms from an elongated germ band into the typical spider appearance. Inversion involves the splitting of the germ band into left and right halves. The two halves separate ventro-medially and move towards lateral and dorsal. As they withdraw from each other, the gap between them is covered by a single layered tissue, which was termed ventral sulcus (Anderson, 1973). This ventral midline epithelium continuously expands while the germ band halves migrate. 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 250h of development in *Cupiennius* and 120h in *Achaearanea* respectively. After dorsal closure when both germ band halves start approaching each other again, this epithelium becomes reduced again and dissolves at ventral closure at the end of embryogenesis at around 350 and 180h, respectively. Embryogenesis is followed by the postembryonic development during which the spiders moult several times until the mature adult emerges (Fig. 1-3; Melchers, 1963; Seitz, 1966; Foelix, 1996).

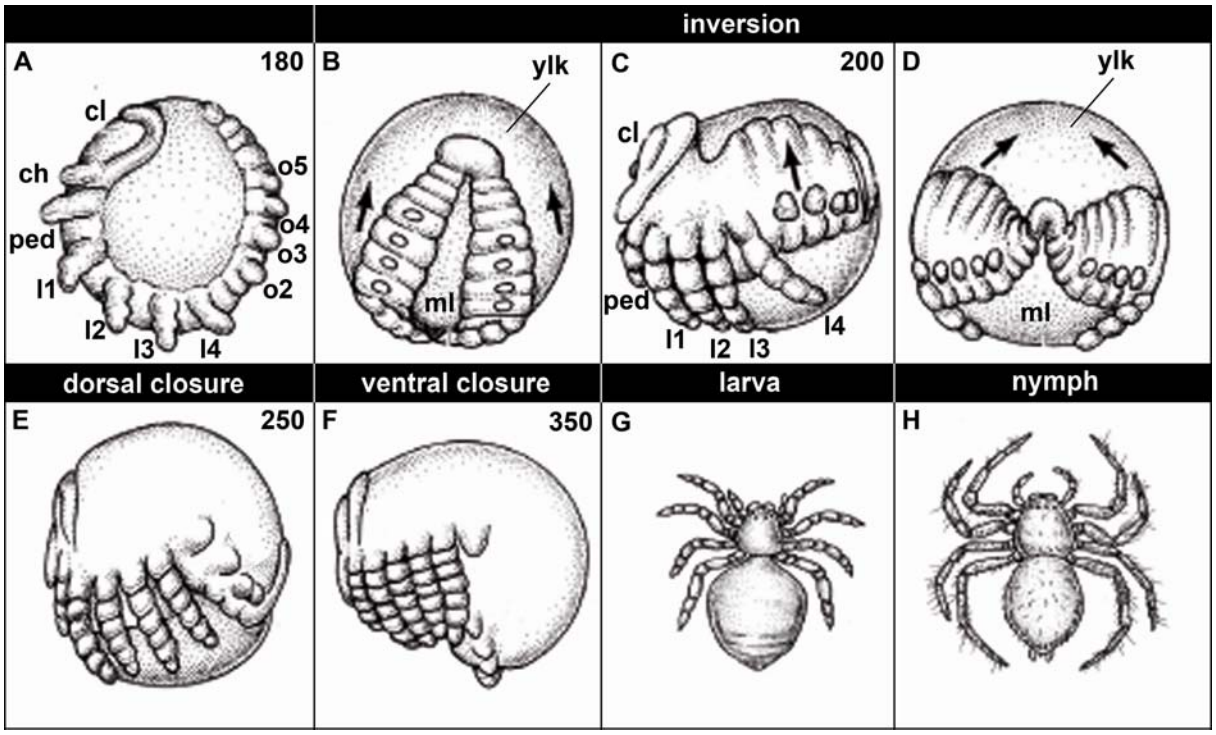

Figure 1-3: Morphological movements during embryonic development of *Cupiennius salei*.

The panel illustrates the morphological movement the embryo undergoes during the process of inversion. (**A**, **C**, **E**, **F**) Lateral view. (**B**, **D**) Ventral view. (**G**, **H**) Dorsal view. (**A**, **B**) In Cupiennius salei it begins at around 180h of development. The left and right germ band halves split at the ventral midline and both sides of the embryo begin to move towards dorso-lateral. (**C**, **D**) During mid-inversion the ventral midline epithelium expands to cover the gap between both germ band halves and the dorsal edges of the germ band migrate further dorsal. (**E**) Inversion ends with dorsal closure at about 250h AEL. (**F**) Embryogenesis is completed at about 350h, when the embryo closes at the ventral side and the ventral midline becomes completely reduced. (**G**, **H**) Embryogenesis is followed by several larval stages. **ch**; chelicerae, **cl**; cephalic lobe, **l1**-**l4**; walking leg 1-4, **ml**; midline, **o2**-**05**; opisthosomal segment 2-5, **ped**; pedipalp, **ylk**; yolk. Modified after Foelix, 1996.

CNS development seems to be similar throughout the chelicerates, at least in basic attributes (Anderson, 1973) and begins when the germ band elongates and the prosomal segments are formed. At first the ventral neurogenic region consists of a single layer of neuroepithelial cells and is positioned medially to the region where the limb buds form in appendage bearing segments and corresponding positions in segments where no such are defined. Proliferation takes place in the apical layers and the newly formed cells invaginate from the apical layers to from so-called neural precursor groups (NPGs). Through the invagination process the neuroepitelium thickens (Stollewerk et al., 2001). These groups start to appear at around 130h AEL in *Cupiennius* and 60h in *Achaearanea* and are formed sequentially in distinct waves at stereotyped positions within the NE (Stollewerk et al., 2001). Eventually, in *Cupiennius* 38 NPGs form and in *Achaearanea* a number of 37 was counted (Döffinger and Stollewerk, 2010). When the primary precursors begin to differentiate

and the number of invagination sites decreases, a second round of mitosis is initiated and secondary invagination sites form (Stollewerk, 2004; Stollewerk et al., 2001).

1.8 Aim

The previous account on arthropod neurogenesis illustrates that early neurogenic events have been analysed to considerable degree in all four arthropod groups and, although there are significant differences observed, were found to be conserved in various aspects. Furthermore, the CNS in adult spiders has been described in some detail and its architecture seems comparable to other arthropod nervous systems. However, axon pathfinding during embryonic development in chelicerates and myriapods has hardly been subject to investigations in the past. So far it has not been described how the nervous system scaffold is formed during embryonic development and comparative data on axonogenesis, as they exist for insects and crustaceans, were not obtained. Hence the aim of this thesis was to fill in the gap in our understanding in these neuroembryological events. A prerequisite therefore was the description of the developing axon scaffold throughout embryonic development of *Cupiennius salei* and *Achaearanea tepidariorum*, two spider species representative for the chelicerates. Based on this information, in a second step I addressed the question, if the establishment of axonal pathways follows a pattern comparable to insects and crustaceans. Furthermore, guidance factors involved in axon pathfinding, such as Netrin, were shown to be highly conserved across species but have not yet been identified in spiders. The study of the expression pattern as well as the functional analysis of *netrins* was therefore part of this thesis. Investigations into the involvement of the ventral midline and the key regulator of midline cell specification in *Drosophila single minded* were conducted to reveal similarities and evolutionary modifications during axonogenesis.

2 Materials and Methods

2.1 Solutions and chemicals

All chemicals used in Mainz have been obtained from the companies MBI Fermentas, Merck, Roth and Sigma-Aldrich, if not stated otherwise. In London chemicals were ordered from VWR. Solutions, buffers and other substances and reagents used can be found in the appendix. All kits used for this thesis have been purchased from Qiagen and handled to the manufacturer's instructions.

2.2 Instruments and equipment

A list of all equipment and instrumentation used can be found in the appendix.

2.3 Animals and animal care

2.3.1 *Achaearanea tepidariorum*

The first cocoons from which the *Achaearanea tepidariorum* (*Achaearanea*) stock was established have been kindly provided by Hiroki Oda's group from Tokio, Japan. In London Alistair McGregor from Vienna, Austria has supported the culture with mated females and juvenile spiders. Adult and juvenile spiders are kept solitary in medium plastic vials at 25°C and 70% humidity, the bottom is covered with humid soil. The vials are closed with foam plugs, which are air permeable. Depending on their size the animals are fed with flies (*Drosophila melanogaster* and *Drosophila pseudoopscura*) or small crickets (*Acheta domesticus*) every second to third day. Mated females make a cocoon every 5 to 6 days of which the first 5 are of good quality. Cocoons contain between 100 to 400 more or less synchronised eggs. The complete lifecycle of *Achaearanea* under laboratory conditions is about 12 weeks.

Cocoons are harvested with a pair of spring steel forceps, opened up and the eggs removed. Cocoons can be closed again using adhesive tape. If kept in a humid chamber, the remaining eggs continue to develop.

Cocoons that are used for breeding are left with the mother until shortly before hatching. Initially hatched spiderlings are kept together in medium plastic vials. After a few moults they are separated and kept solitary.

2.3.2 *Cupiennius salei*

For the colony in Mainz *Cupiennius salei* (*Cupiennius*) mated females have been kindly provided by Prof. Ernst-August Seyfarth (Frankfurt am Main, Germany). In London a breed has been established using animals, which came from the same source. Adult individuals are kept in large cucumber jars at 25°C and approximately 70% humidity. The bottom of each glass jar is covered with moist soil, the jar opening closed with a permeable lid. Each jar is provided with a small water container. The spiders are fed with crickets twice a week, newly mated females every second day. Fertilised females produce a cocoon, which they carry with them, every three to four weeks approximately, up to five cocoons altogether. *Cupiennius* cocoons can contain up to 1000 eggs. Only the first three are usually used to maintain the culture or provide embryos for experiments. The lifecycle of *Cupiennius* takes 9 to 12 months from fertilised egg to adult spider.

For analysis cocoons are taken from the female, which is anaesthetised using CO_2, with long forceps. As is the case in *Achaearanea*, cocoons can be opened and resealed again, the embryos continue to develop.

To maintain the culture, cocoons are left with the mother for about 8 days. Newly hatched spiders are kept together until after a few moults. They are then separated into individual glass jars. During development they are fed with *Drosophila melanogaster* and *Drosophila pseudoobscura* and different sizes of *Acheta domesticus*.

2.4 Biomolecular methods

2.4.1 Working with RNA

To prevent degradation of RNA it is necessary to follow certain guide lines. It should always be made sure that working with RNA is handled under sterile conditions and with sterile equipment. Solutions should be RNase free and the activity of endogenous enzymes is suppressed by keeping the samples on ice.

2.4.2 RNA-Isolation

Total RNA of *Cupiennius* and *Achaearanea* is isolated from different embryonic stages using the TRIZOL protocol (Invitrogen). An average volume of about 250µl of embryos is used per isolation. Embryos are obtained from the cocoon, dechorionised as described later (2.6.2). All water is removed and the embryos are used immediately or stored at -80°C for later preparation of RNA.

To prepare RNA, the embryos are crushed in a small volume of TRIZOL in a cap (2ml for *Cupiennius* and 1.5ml for *Achaearanea*) using a pistil, homogenised in a total volume of 750µl of TRIZOL and incubated at room temperature (RT) for 5min. 20µl of chloroform is added to the homogenate, the cap shaken vigorously and then left standing at RT for another 3min. The homogenised embryos are centrifuged for 15min at 13000 rounds per minute (rpm) and 4°C, the supernatant (aqueous phase) is carefully transferred to a fresh 1.5ml cap and the RNA precipitated by mixing the solution with 500µl 100% isopropanol. After incubation for 10min at RT, the sample is centrifuged for 15min at 4°C and 14000rpm. The supernatant is removed on ice and the pellet washed with 500µl of ice cold 70% DEPC-EtOH in another 4°C centrifugation step for 5min. After this, the supernatant is discarded and the pellet dried in a speed vacuum manifold until all traces of alcohol are evaporated. The RNA is dissolved in 50µl of DEPC-H_2O and 1µl of RNase Inhibitor is added to prevent degradation. The RNA is checked on either a RNA gel or on an agarose gel and stored at -80°C and the rate of yield is determined using a photometer.

2.4.3 Reverse transcription of RNA

The cDNA is generated using reverse transcriptase (Superscript III, Kit from Invitrogen) that uses RNA as a template. Either Oligo dT or Random primer can be used for the reaction, but better results were received using Oligo dT.

To generate cDNA from total RNA, 5µg RNA are mixed with 1µl Oligo dT primer (3µg/µl) and filled up to a volume of 10µl with DEPC-H_2O. This is followed by an incubation step for 10min at 72°C and then the sample is cooled down to 4°C. 4µl first strand buffer (5x), 4µl dNTP's (5mM), 1µl DTT (0.1M) and 1µl of RNase inhibitor are added, mixed and incubated for 2min at 42°C. 1µl of Superscript III (200u) is added to the mixture, another incubation step follows, this time for 50min at 42°C. Heat inactivation of enzyme activity is facilitated at 72°C for 10min. Finally, remaining RNA template is degenerated by adding 1µl of RNase H and incubation for 20min at 37°C. The cDNA can be stored at -20°C.

2.4.4 PCR

The PCR (polymerase chain reaction) is utilised to amplify specific DNA sequences from cDNA templates using DNA polymerase. Specific primers are designed, if the desired DNA sequence is already known. Degenerate primers are useful to amplify unknown gene sequences. These are complementary to specific conserved sequences of homologous genes in other species.

2.4.4.1 Standard PCR reaction

All PCR reactions are carried out in a total volume of 30µl. Standard PCR reactions for the initial PCR contain 2µl cDNA, 1-2u Taq polymerase, 1x Taq polymerase buffer, 12.5mM 3´primer, 12.5mM 5´primer, 0.6µl 10mM dNTPs and double distilled water (ddH_2O).

For nested PCR reactions that should yield a more specific product, the same contents are used, the only difference is, that instead of cDNA, 1µl PCR template from the initial PCR is used.

A typical PCR protocol contains the following steps:

1 95°C for 60sec
2 95°C for 30sec
3 56-63°C for 60
4 68°C for 90sec
5 68°C for 5min
steps 2-4 are repeated 30 times.

95°C ensure the complete denaturisation of the cDNA template. The annealing temperatures in the third step depend on the length and GC-content of each primer and are chosen accordingly to the individual primer combinations used. 68°C is the optimum temperature of Taq polymerase to synthesise and elongate new DNA strands.

Name	Sequence (5' - 3')	length	Tm °C
cloning primer			
M13 forward	CTGGCCGTCGTTTTACA	17	50.70
M13 reverse	CAGGAAACAGCTATGAC	17	57.60
***Cs netrin* degenerate primer**			
Net1	GNMGNTGYATHCCNGAYTTYG	21	62.24
Net2	GTNACNYTNACNYTNWSNYTNGG	23	62.77
Net3	CCNGTNYTNACNYTNWSNYTNGG	23	65.44
Net3b	GAYTGGGTNACNGCNACNGA	20	63.48
Net3re	GTNACCCARTCYTGNARNACAGG	23	65.44
Net3bre	TCNGTNGCNGTNACCCARTC	20	63.48
Net4	GTNGGNGGNMGNTGYAARTG	20	63.48
Net4re	CAYTTRCANCKNCCNCCNAC	20	63.48

Net4b	TGYAARTGYAAYGGNCAYGC	20	60.40
Net4bre	GCRTGNCCRTTRCAYTTRCA	20	60.40
Net5re	TGYTTRCANGGRCAYTGNCC	20	62.45
Net5bre	CCNGTNACNCCRTCYTTRCA	20	62.45
***Cs netrin* specific primer**			
Net1sp	GGTGTATACCGGATTTCGTC	20	60.40
Net1/2sp	GGTGCATACCGGATTTCGTC	20	62.45
Net1/3sp	CGGATTTCGTCAACGCTGC	19	62.32
Net2sp	GCACAACGTGTCTCTGACACTG	22	64.54
Net3sp	GCCCTGTTCTTCAAGACTGG	20	62.45
Net3bsp	CAAGACTGGGTGACGGCAAC	20	64.50
Net3resp	GTCACCCAGTCTTGCAGCACTGG	23	68.12
Net3bresp	GTTGCCGTCACCCAGTCTTGC	21	66.47
Net4sp	GGAGGCAGATGCAAATGCAATG	22	62.67
Net4bsp	CAAGACTGGGTGACGGCAAC	20	64.50
Net4resp	CCATTGCATTTGCATCTGCC	20	60.40
Net4bresp	GGCATGTCCATTGCATTTGCATC	23	62.77
***At single minded* specific primer**			
Atsim_1	GNATGAARTGYGTNYTNGC	19	58.00
Atsim_2	TANSWYTGNACCCANACCCA	20	60.40
Atsim_3	CCNSWRCARTGDATNACYTT	20	58.01
sim_f1(1)	GGATGAAATGCGTTCTGGC	19	60.16
sim_f2(2)	TATCACTACATTCACGCCTC	20	58.35
sim_f3	GAGTTGACAGGCGACAGTTGC	18	62.18
sim_f4	GCGCCCCCATATGACAGTTGC	21	66.47
sim_f5	CGAGCCAACATGGATCTCAG	20	62.45
sim_re1(3)	AAGGTCCGACGCTATTCGTA	20	60.40
sim_re2	GCTGCTGAAAGGTCCGACG	20	64.48
sim_re3	GCTGTGACTATTACCACTGCTGC	23	64.55

Table 2-1: List of primer used for specific and degenerate PCR.

Name	Sequence (5' - 3')	length	Tm °C
RNAi primer			
AtnetRNAi_1for	CATGGACTACGGAAAGACAT	20	59.00
AtnetRNAi_2for	CAGTCAGTGCCGGAAGATGT	20	65.40
AtnetRNAi_1re	GTTAGCCTTTGTAATGGCGG	20	63.20
AtnetRNAi_2re	GCACCTGTTATGGGCTCAAC	20	64.40
AtnetRNAi_3re	CCTTCCAGAGTGCTAAACGC	20	63.80
AtnetRNAi_4re	CTTTAATGTCGGTTGCCGTC	20	64.20
AtsimRNAi_1for	GCTTGGTCAGTAATGGGATGA	21	63.80
AtsimRNAi_1re	CTCGCTAGCCTGGATGTTCT	20	63.40
AtsimRNAi_2for	CCTTATATTGCGGTTGCCAT	20	63.40
AtsimRNAi_2re	CGAGCAGCATTTTTGGATTT	20	63.90
AtsimRNAi_3for	GCCATTCTCAATTAAGGGCA	20	63.70
AtsimRNAi_3re	CAGCGTTTTCTTTCTCTCGG	20	63.90

Table 2-2: List of primer used for generating fragments for RNA interference.

2.4.4.2 Colony PCR

To analyse, which of the colonies grown after a transformation (2.5.4) on the plates carry the plasmid with the designated insert, we use colony PCR. Between 24 and 96 single colonies are analysed simultaneously. One PCR reaction mix contains 1x Taqbuffer, 1µl dNTPs (2mM), 1µl M13 forward primer (10mM), 1µl M13 reverse primer (10mM), 0.2u Taq polymerase and is added up with ddH_2O to a final volume of 10µl. A master mix for all colonies to be picked is prepared and distributed to an equivalent number of PCR tubes. The individual colonies are picked with a tip, dipped into the PCR reaction mix and streaked onto a kanamycin containing (50µg/ml) replication plate that is partitioned into a numbered grid.

PCR program:

1 96°C for 10min
2 94°C for 60sec
3 55°C for 30sec
4 72°C for 60sec
5 72°C for 7min
steps 2-4 are repeated 35 times.

The PCR samples are checked with a DNA gel (see 2.4.6.1). Positive colonies that have incorporated the plasmid can be estimated on the basis of their size.

2.4.5 DNA purification

PCR fragments are either purified by using the “PCR Purification Kit” from Qiagen following the manufacturer’s instructions or by doing a phenol-chloroform extraction. If the PCR yields more than one band during amplification, the desired band is cut out from the gel and the DNA either extracted using the “Gel extraction Kit” (Qiagen) or manually, performing a phenol-chloroform extraction.

2.4.5.1 Phenol-Chloroform extraction

To degenerate enzymes and remove these and other contaminations from a DNA solution the DNA containing solution is pipetted to a precentrifuged phase lock cap (Eppendorf). The same volume of phenol-chloroform is added and the mixture shaken vigorously, so that containing proteins are degenerated and can then be found in the organic phase. To separate the phases the cap is centrifuged for 5min at RT and 14000 rpm. During this the heavier, organic phase moves through the gel into the lower part of the cap. The lighter, aqueous phase, containing the DNA, remains on top of the gel. An equal volume of chloroform is added to the upper phase and the cap is shaken and centrifuged as before. This step is repeated once to eliminate all traces of phenol. Finally the aqueous phase above the gel can be transferred to a fresh cap and precipitated.

2.4.5.2 Precipitation of nucleic acids

Then also traces of salt or other substances (such as isoamyl alcohol) have to be removed from the DNA solution. Therefore we precipitate the DNA with 1/10 of the volume of sodium acetate (3M, ph 5.2) and 2 ½ volume of ethanol (100%). Precipitation takes place over night (ON) at -20°C. For precipitation of RNA, LiCl (4M) is used as salt. The next day the solution is centrifuged for 30min at 4°C and 14000 rpm. The supernatant is discarded and the pellet washed with cold 70% ethanol or 70% DEPC-ethanol respectively by centrifuging the cap for 15min. All supernatant is removed. The pellet is dried either in a vacuum centrifuge manifold or in a heat block at 42°C and can then be solved in either EB buffer, ddH_2O or DEPC-H_2O.

To determine the concentration of nucleic acids, samples are measured photometrically or analysed on an agarose gel.

2.4.6 Gel electrophoresis

The technique is used to separate DNA or RNA fragments according to their length using agarose gels and electric current. The agarose molecules interlink in the gel, the higher the concentration the tighter cross-linked is the gel. For larger fragments one would choose gels of a lower percentage and use a higher concentrated gel for smaller DNA fragments. Negatively charged nucleic acid molecules migrate towards the positive pole in the energised gel (between 70 and 120V).

2.4.6.1 DNA gel electrophoresis

For a DNA gel the required amount of agarose is added to 50ml 1xTEA. To check on PCR samples etc, a 0.75% agarose gel is used and run at 95mV. To separate fragments of similar length and those that should be used for gel extractions, 1.5% agarose gels are used. The mixture is boiled until the agarose is completely dissolved and then left standing to cool down before 20µl of ethidium bromide (EtBr, 1mg/ml) are added to the gel. EtBr intercalates in-between the DNA bases and accumulates within the molecule. It is fluorescent under UV light, so that the DNA

fragments are visible. The mixture is transferred to a gel slide with a comb, which moulds pockets into the gel where later the samples will be filled into. When the agarose gel is hardened, the comb is removed and the samples can be loaded onto it. 6x loading dye is added to the probes. This enhances the density of the probes and the containing dye makes it easier to track the migration of the probes within the gel. 1x TEA is used as running buffer. To determine the sizes of the different DNA fragments a DNA ladder with fragments of known size is also loaded on to the gel. We either use a 100bp+ ladder (Fermentas) or 2-log base pair ladder as marker.

2.4.6.2 RNA gel electrophoresis

RNA gels are used to analyse ribonucleic acid. Beside agarose they also contain formaldehyde to create enzyme inactivating conditions that prevent degradation of the RNA sample. RNA gels we use contain the required amount of agarose in 30ml DEPC-H_2O and 4ml 1x MOPS. The mixture is boiled until the agarose is dissolved. After cooling, 6.8ml 37% formaldehyde are added, the liquid filled into the slide and left standing to harden. Before the samples are loaded onto the gel they are incubated with loading buffer at 70°C for 5min to denaturise the RNA. RNA loading buffer contains EtBr to visualise the bands under UV light. After the incubation step, loading dye is added and the gel run as described before (see above, 2.3.6.1).

2.4.7 Gel extraction of PCR fragments

To separate differently sized fragments of one PCR reaction, we use the gel electrophoresis as described before. The desired bands are cut out from the gel using a scalpel. Extraction and purification of the PCR fragments is either achieved by using the “QIAQuick Gel Extraction Kit (250)“ from Qiagen or via a phenol-chloroform extraction using phase lock gel caps (2.3.5.1). This second procedure is followed by an ethanol precipitation of the DNA (2.3.5.2).

2.4.8 *In vitro* transcription

To detect where the transcript of a specific gene of interest is expressed in a tissue or organism, DIG (digoxigenin) labelled antisense RNA probes are generated to be used for *in situ* hybridisations (*in situ*). RNA probes are made using a technique called *in vitro* transcription. RNA polymerase is the enzyme, which transcribes a complementary RNA probe from a cDNA template. RNA polymerases start the transcription from a specific promoter which lies adjacent to the insert. Therefore the orientation of the insert within the plasmid determines which polymerase can be used to generate an antisense probe. Labelling of the probe is enabled through the use of DIG labelled UTPs that are integrated into the newly synthesised RNA probe.

For the reaction 1µg of cDNA template, 2µl transcription buffer (10X,), 2µl rNTP DIG labelling mix (Roche), 2µl RNase inhibitor (20u) and 2µl RNA polymerase (40u, T7, SP6, Roche) according to the orientation of the insert are prepared and filled up with DEPC-H_2O to a final volume of 20µl. The mixture is then incubated for 2h at 37°C. To digest any unprocessed cDNA, 2µl of RNase free DNase I are given to the sample, mixed and left on 37°C for another 15min. All enzymatic reactions are stopped by adding 2µl of 0.2 EDTA to the probe solution and letting this stand for a couple of minutes. For purification of the generated RNA probe, the sample is processed as described under 2.3.5.2. The RNA probe is dissolved in 50µl DEPC-H_2O and 1µl RNase inhibitor is added to prevent the probe from degrading. A test gel is run and the RNA probes are stored either at -20 or -80°C.

2.4.9 Generation of double stranded probes for RNA interference

RNA interference is a relatively new technique to specifically down-regulate gene function (Baulcombe, 1999; Sharp, 1999). A short (about 100 nucleotides), double stranded (ds) RNA complementary to the gene sequence is synthesised. This longer sequence is cut into shorter fragments (siRNA, ca 20 nucleotides) by a so called dicer, an RNase III nuclease and are then integrated into RNA protein complexes, named RISC. This reaction is comparable to the immune response of an organism to

viral infections (Bernstein et al., 2001). The down-regulation happens when RISC bind to complementary mRNA sequences, whose degradation they induce. To reduce off targets, ds RNAs should not be longer than 100-200bp, the sequence should be positioned close to the 5´ end of the gene but must not cover the first 100bp of it (Qiu et al., 2005).

Fragments that fulfil the required conditions are amplified and cloned. The generation of ds RNA in principle is an *in vitro* transcription with the difference that instead of only one RNA polymerase both T7 and Sp6 polymerases are used in one reaction. Unlabelled nucleotides are used to assemble the two strands. The purified and tried pellet is dissolved in 10µl DEPC-H_2O to increase the concentration. The ds RNA is then heated to 80°C for 5min and slowly cooled to RT to allow exact annealing of the complementary strands. Double stranded RNA is stored at -20°C until needed.

2.5 Microbiological methods

Cloning is a strategy to integrate a defined DNA sequence into a vector plasmid, which then is inserted into bacterial cells. Together with the bacterial genome the inserted vector is amplified exponentially. The vector itself bears features that allow not only a directed amplification of the desired fragment but also enables us to directly generate RNA probes or double stranded RNA constructs complementary to the sequence of interest. For all cloning experiments we use the “pZERO-2 Cloning Kit” purchased from Invitrogen. For the transformation of the vector plasmid into bacterial cells we use either electrocompetent or chemically competent E.coli cells.

2.5.1 Vector and Vector preparation

A map of the vector pZero used can be found in the appendix. Before the DNA fragments can be integrated into the vector, the plasmid itself has to be linearised. Restriction endonucleases are enzymes that cut DNA into exactly defined fragments. They recognise specific sites within a given DNA sequence and induce double strand brakes, which either lead to blunt or cohesive ends. For this work we use EcoRV to digest pZero and facilitate blunt ends. A 10µl reaction mix contains 1µl pZero stock,

1µl EcoRV, 1x enzyme buffer and is incubated at 37°C for 30min. To stop the enzymatic reaction and remove all of the enzyme and other components, the restriction mix is purified using a phenol-chloroform extraction with following ethanol precipitation (2.3.5.1 and 2.3.5.2). The pellet is dissolved in 90µl ddH_2O and stored at -20°C. 1µl of the restricted vector is checked on a gel.

2.5.2 Preparation of the insert

To ligate the DNA fragment into the vector the fragment also has to have blunt ends. We use Klenow fragment, which fills in recessed 3‘ ends and digests protruding 3‘ overhangs to produce blunt ends. A 60µl reaction mix contains 50µl PCR product, 1x Klenow buffer, 4mM dNTPs, 2u Klenow fragment and ddH_2O and is incubated for 40min at 37°C. The digested PCR product is checked on a gel. If several fragments show a similar size, the whole PCR sample is run on a highly concentrated gel to separate individual bands. The desired fragment is then cut out and purified as described in paragraph 2.3.7. If the gel shows one band of the right size, samples can be purified directly using the “PCR purification Kit” from Qiagen or phenol-chloroform and ethanol precipitation (2.3.5.1 and 2.3.5.2).

2.5.3 Ligation

As bacteria can only integrate circular DNA molecules into their genome, it is necessary to ligate linearised fragments of both vector and PCR fragment at their blunt ends to obtain circular DNA molecules. This procedure is called ligation and is catalysed by the enzyme T4 ligase. A typical ligation reaction contains 7.5µl of purified PCR product, 1µl of linearised pZero vector, 1x ligation buffer and 2.5u T4 ligase. Sterile water is added to a final volume of 10µl. The reaction is carried out at 16°C for one hour and the probes are either directly used or stored at -20°C.

2.5.4 Transformation of electrocompetent cells

During transformation exogenous DNA is transported into cells, so that the plasmid will be amplified with the bacteria. The transformation of electrocompetent E. coli is performed using a procedure called electroporation. Here a high voltage pulse is administered to the cells so that the DNA is incorporated by the cells.

Electrocompetent TOP10 cells (50µl, Invitrogen) are left to thaw on ice, 2µl of the ligation product is added and the suspension is transferred to a sterile cuvette. The cuvette is then charged with 2kV/ms. Immediately after the transformation 250µl warm SOC medium are added to the cells and they are incubated at 37°C for 1h to initiate growth and the kanamycin resistance. The cells are then plated onto a pre-warmed LB-Kan plate (50µg/ml Kanamycin) and incubated ON at 37°C. The next day colonies can be picked for further analysis.

2.5.5 Transformation of chemically competent cells

With this technique plasmids are transferred into bacteria with the help of a heat shock. Chemically competent TOP10 (50µl, Invitrogen) cells are slowly thawed on ice and mixed with 4-10µl of the ligated plasmid. Before the heat shock is administered at 42 °C for 2min, they are incubated for 30min on ice. Directly after the heat shock the cells are put back on ice and 250µl SOC medium are added. The growing and plating of the cells is accomplished as is described for electrocompetent cells.

2.5.6 Culturing bacteria and preparation of plasmid DNA

Positive tested clones (see colony PCR, 2.3.4.2) are picked from the replication plates and inoculated in 4ml Kanamycin containing LB (50µg/ml) medium ON at 37°C on a shaker. The next day 3ml of the ON culture are pelleted at 3000 rpm for 5min. Plasmids are isolated using the “Qiagen Mini Prep-Kit” according to the manufacturers instructions. The protocol is based on the principal of alkaline lysis. Here cells are disintegrated using a detergent (usually SDS). The bacterial ring

chromosome is attached to the cell membrane and can be pelleted together with the cell debris. After centrifugation the plasmid DNA is found in the supernatant, which then is purified by using the provided columns. The DNA is eluted from the column with 50µl pre-warmed EB buffer. The concentration of the plasmid DNA is calibrated either by using a photometer or on a gel.

2.6 Micromanipulation

For embryonic RNAi experiments double stranded RNA is injected into the embryo prior to the formation of the embryonic germ band. In *Cupiennius* this is at the third day after the cocoon (after egg laying, AEL) has been made. *Achaearanea* embryos are injected on the second day AEL.

2.6.1 Capillaries

For the injection fine glass capillaries (GB 100-TF 8P, Science Products GmbH) are pulled in a vertical puller, resulting in a rather long, fine tip.

2.6.2 Preparation of the embryos

The cocoons are taken from the mother, opened up, the embryos dechorinated with chlorine bleach (6%) for 2min and then washed thoroughly with tap water. They are transferred to a Petri dish filled with coloured agar, arranged in rows and columns with a tissue paper of which one corner is twirled. The embryos are left to dry for about 10min to reduce the inside pressure of the embryo. A cover slip (18mm x 24mm for *Cupiennius*, 18mm x 18mm for *Achaearanea*), coated with heptane glue, is taken and with that the embryos are lifted from the Petri dish. Again, they are left to dry for 10min before transferring the cover slip to a small Petri or a weighing dish, were they are covered with 3A Voltalef oil so that the embryos are almost completely immersed (Fig. 2-1).

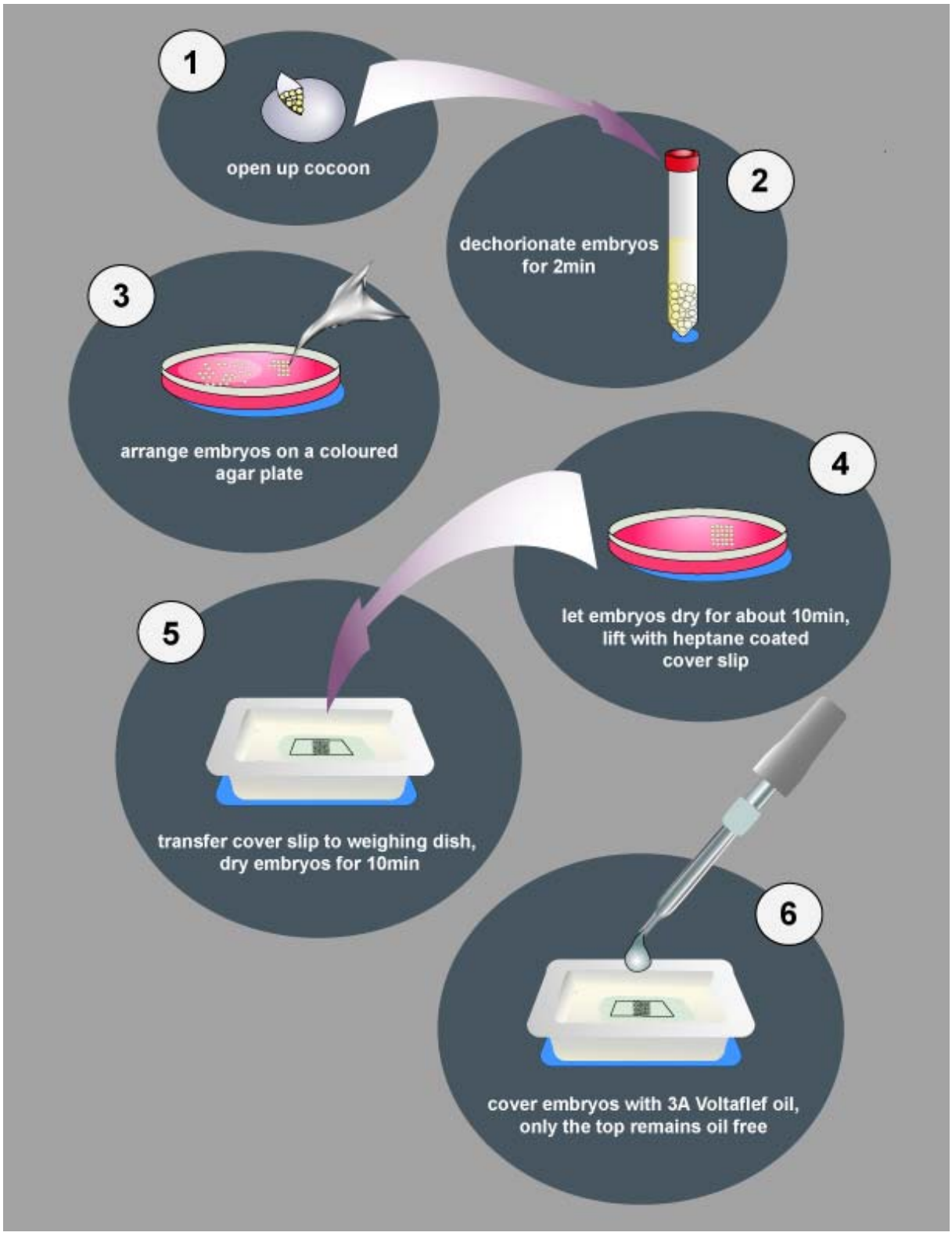

Figure 2-1: Preparation of spider embryos for RNAi injection.

The description of the single steps can be found in the text under paragraph 2.5.2, the figure is modified after Christof Rickert.

2.6.3 Injection procedure

The injection procedure is performed using a micromanipulator connected to a FemtoJet injection unit (Eppendorf). A capillary is loaded with 2µg ds RNA of the either the specific gene or GFP control and broken with forceps prior to injection. The injection solution is mixed with food colouring to allow the tracing of injected material in the embryo. The angle of the capillary should be as steep as possible, so that the embryos can be injected into the top region, which is not covered with oil. After injection the embryos are completely covered with 3A Voltalef oil and kept in a humid chamber at 25°C until they reach the desired developmental stage for further analysis (Fig. 2-2). Before fixation the embryos are removed from the cover slip using heptane.

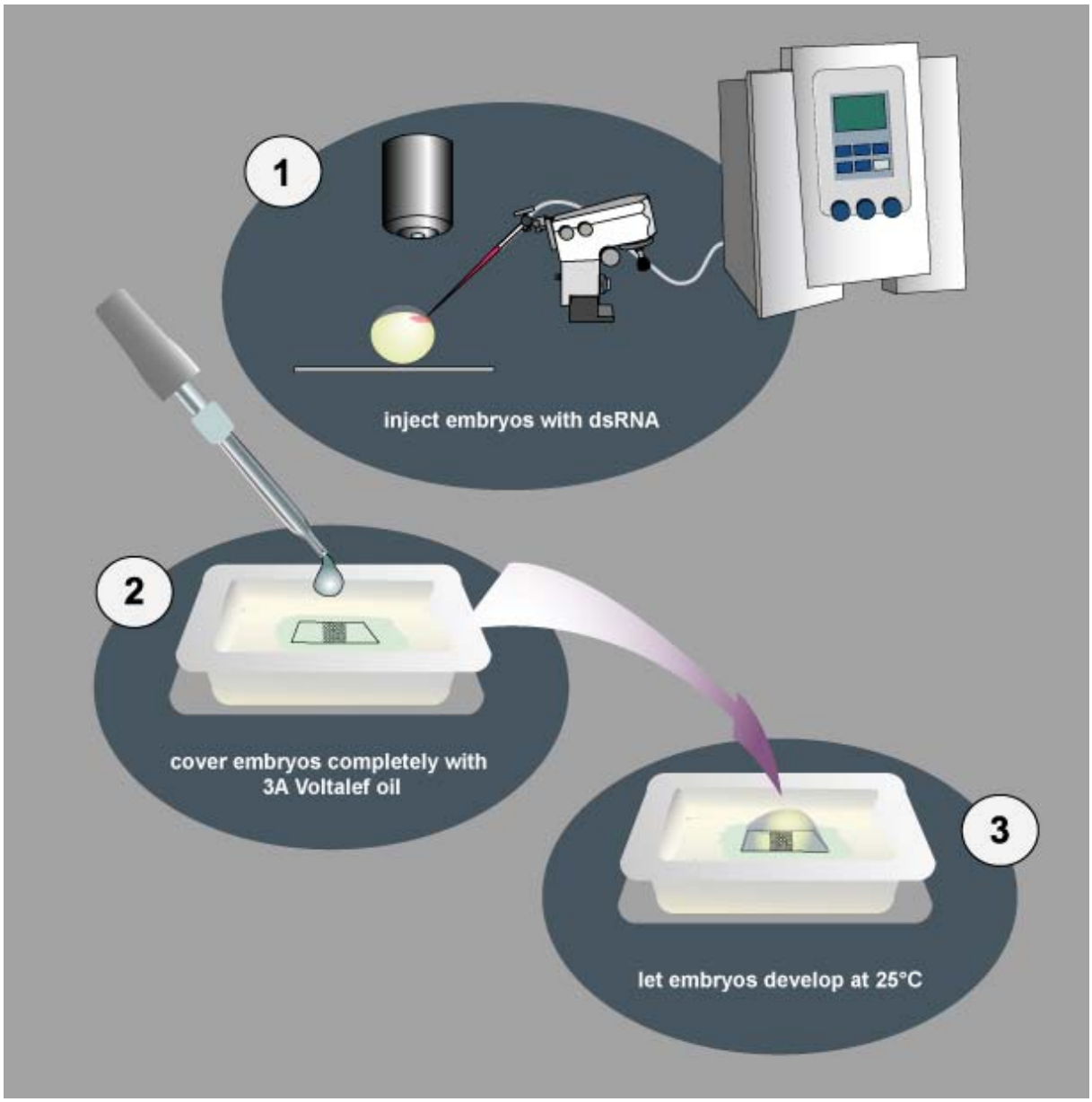

Figure 2-2: Experimental set up and steps after injection.

The description of individual steps can also be found in the text under paragraph 2.5.3, the figure is modified after Christof Rickert.

2.7 Immunohistochemistry

2.7.1 Fixation

To prepare spider embryos for Phalloidin, antibody staining and *in situ* hybridisation first of all the chorion has to be removed. Embryos are taken from the cocoon and transferred to 15ml falcons. 6% chlorine bleach is used to chemically remove the egg

membrane. After incubating the embryos with bleach for 2min, they are washed thoroughly with tab water. Shortly before fixation all water is removed.

Embryos of both *Cupiennius* and *Achaearanea* are fixed in a two-phase solution of 1 volume heptane and 1 volume 5.5% formaldehyde in PEMS. Normal MeOH-stabilised formaldehyde is used for *in situ*s, the embryos are fixed at RT on a shaker or wheel ON. For Phalloidin and antibody stainings methanol free formaldehyde (Polyscience, Inc.) is used. Here fixation takes place 1-2 hours at RT or ON at 4°C.

After the fixation the lower half of the fixative is removed and replaced by either MeOH or EtOH, according to the formaldehyde used and lightly shaken to brake up the vitelline membrane. Then all of the liquid is replaced by 100% MeOH or EtOH respectably and washed with several changes of the solvent for 1 to several hours. The embryos are then stored at -20°C.

2.7.2 Devitellinisation

The vitelline membrane hinders the permeation of Phalloidin, antibodies or RNA probes. It has to be removed manually using fine, sharp forceps.

2.7.3 Phalloidin staining

Phalloidin is one of a group of toxins from the death cap (*Amanita phalloides*) known as phallotoxins. It binds specifically at the interface between F-actin subunits, locking adjacent subunits together and can be labelled with fluorescent analogs. Since F-actin is a component of the cytoskeleton, phalloidin stainings are helpful to visualise cell shapes under a fluorescent microscope.

For Phalloidin stainings embryos are stepwise transferred from 100% EtOH to PBS. Then the embryos are blocked in 1% BSA in PBS for 1h at RT to reduce background staining. A 10µl Phalloidin-FITC aliquot is vaporised and resolved in 200µl PBS with 1% BSA. The embryos are incubated with the Phalloidin solution in the dark at 4°C

for up to 48h. To remove the Phalloidin solution the embryos are rinsed and washed with PBS for several times. All steps are carried out on a shaker and in the dark. Finally all PBS is removed and the embryos are transferred to a 1:1 solution of 70% glycerol in PBS/ Vectashield mounting medium (Vector laboratories, Inc.). Phalloidin is not very stable, stainings can be kept for short periods at -20°C.

2.7.4 Antibody staining

Antibody stainings are used to visualise the expression pattern of a specific protein within a given tissue. The primary antibody binds to a specific epitope and can be detected via a labelled secondary antibody, which in turn binds specifically to the primary one. To make the tissue permeable detergents are used in the washing buffer. However, since detergents reduce the quality of a Phalloidin staining, the concentration of it in the wash buffer should be as low as possible whenever an antibody staining is combined with a Phalloidin staining. Usually 0.02% PBTween is used.

In general for an antibody staining fixed embryos are transferred from EtOH into 0.3% PBT with 0.1% BSA (called PBT) by several rinsing in 1x PBS (PBS) and washing steps with PBT (3x 10min, 4x 30min). To sustain better staining results embryos sometimes are flat prepped, meaning that the legs and most of the yolk is removed during the washing steps. Then the embryos are blocked in PBT+N (PBT+NGS 5%) for 2x 30min to reduce background staining. Primary antibodies are diluted in PBT+N (see table 2-2) and incubated ON or up to 48h at 4°C. The first antibody is removed and in case of *Cs* Prospero and *At* Cadherin stored at 4°C for later reapplication. The embryos are rinsed several times with PBT and washed again in 4x 30min in PBT and 2x 30min in PBT+N. The secondary antibody is incubated ON at 4°C. If a secondary antibody labelled with a fluorescent dye is used, incubation and all subsequent steps are done in the dark. The next day the antibody is removed and the embryos are rinsed in PBT and washed 4x 30min in PBS. All incubation and washing steps are operated on a shaker. The embryos are then mounted to 70% glycerol in PBS or a 1:1 mixture of 70% glycerol and Vectashield Mounting medium. The stainings can be stored at -20°C.

2.7.5 Antibodies used

The in this thesis used antibodies are listed in the following tables (tables 2-3 and 2-4.). The primary antibodies anti-*At* Cadherin (Cadherin) and anti-*Cs* Prospero (Prospero) can be used several times. 0.01% sodium azide is then added to the aliquots to prevent infestation with micro organisms. Anti-acetylated α Tubulin (Tubulin) is only once.

Name	Species	Dilution	Origin
primary antibody			
anti-acetylated α-Tubulin	mouse	1:100	Sigma
anti-*At* Cadherin	guinea pig	1:100-200	Oda
anti-*Cs* Prospero	rat	1:200	Weller/ Tautz
anti-DIG alkaline phosphatase	sheep	1:1000	Roche

Table 2-3: List of primary antibodies used.
The listed antibodies are used in the stated concentrations.

Name	Label	Species	Dilution	Origin
secondary antibody				
anti-guinea pig	FITC	donkey	1:500	Dianova
anti-guinea pig	Cy3	donkey	1:500	Dianova
anti-guinea pig	Cy5	donkey	1:500	Dianova
anti-mouse	Alexa	donkey	1:500	Dianova
anti-mouse	Cy3	donkey	1:500	Dianova
anti-mouse	Cy5	donkey	1:500	Dianova
anti-rat	Cy5	donkey	1:500	Dianova

Table 2-4: List of secondary antibodies used.
The listed antibodies are used in the stated concentrations.

2.7.6 *In situ* hybridisation

In situ hybrisations allow not only to analyse the spatial expression of a specific gene transcript but also the temporal component of gene expression. To detect the mRNA in the tissue DIG labelled antisense RNA probes are used, that bind complementary to the transcript of interest. The DIG label in turn is recognised through an antibody that carries alkaline phosphatase to catalyse the staining reaction. *In situ* hybridisations should be realised under sterile conditions since RNA probes are handled.

Embryos from the -20°C storage are transferred from MeOH to 0.1% PBTween (PBTw) by washing them in 50 and then 30% MeOH, both times for 5min and 5min in PBTw. This is followed by a post fixation with 140µl formaldehyde (37%) in 1ml PBTw for 20min, a washing step in PBTw for 5min and incubation with proteinase K (2.5µl 1:10 dilution in 1ml PBTw) for 5min. This is washed off again with PBTw for 5min before a second postfixation step follows as described above. After that the embryos are washed twice for 5min in PBTw and then once in DEPC-H_2O. Next they are incubated with 1ml TEA and 2.5µl acetic anhydride for 1h. The embryos are stepwise transferred to hybridisation solution B (hyb B) by washing them twice in PBTw for 5min, then 5min in 1:1 PBTw/ hyb B. All previous steps are carried out on a shaker and at RT. The following steps are conducted on a thermo shaker at 65°C. Hyb B is substituted by hybridisation solution A (hyb A) and prehybridised for 4h. The mRNA probes are diluted with hyb A according to table 2-5, heated to 100°C for 5min and cooled down on ice. This ensures that the probe is single stranded and does not contain any secondary structures, which might hinder the annealing with the complementary sequences in the tissue. Hybridisation takes place at 65°C for a minimum of 18h.

RNA probe	Label	Dilution
Cupiennius netrin homologue (*Cs net*)	DIG	1:250
Cupiennius islet homologue (*Cs isl*)	DIG	1:250
Achaearanea netrin homologue (*At net*)	DIG	1:250
Achaearanea single minded homologue (*At sim*)	DIG	1:250

Table 2-5: List of antisense RNA probes.
The listed RNA probes are used in the stated concentrations.

The next day the probe is removed and stored at -20°C for reuse. The embryos are washed in hyb B with 25%, 50% or 75% 2x SSC and 2x SSC each for 15min. This is followed by two washes for 30min with 0.2% SSC. All wash buffers used until here are preheated to 65°C. The following steps are carried out at RT again and comprise washes in 0.2% SSC with 25%, 50% or 75% PBTw and PBTw, each step is 10min. Afterwards the embryos are blocked for about 2h with 2% sheep serum in PBTw. The antibody (anti-DIG AP) is diluted in PBTw and incubated ON at 4°C.

On the third day the antibody is removed and discarded. The embryos are washed 8x 15min in PBTw and twice for 5min in AP detection buffer at RT. The staining reaction is conducted in the dark and observed at regular intervals. The staining solution contains 3.5µl BCIP and 4.5µl NBT in 1ml AP buffer. If necessary the solution is exchanged repeatedly. Once the staining is strong enough the reaction is stopped by rinsing the embryos trice in PBTw and fixing them in 140µl formaldehyde (37%) in 1ml PBTw for 20min to inactivate the alkaline phosphatase. Several washes with MeOH remove the reddish tint of the *in situ*. This step was only performed if no antibody staining followed. Stained embryos can be stored in MeOH and for longer time spans in 70% glycerol in PBS at -20°C.

For a following antibody staining with Tubulin or Cadherin the MeOH step is left out since MeOH can affect the characteristic of cell membrane components. The embryos are directly transferred to PBT by rinsing and several washing steps. The antibody staining is carried out as described before (2.8.3).

2.8 Preparation, documentation and analysis of stainings

2.8.1 Whole mount embryos

Whole mounts are transferred from the cap to a black glass dish filled with 70% glycerol and arranged in a way the staining can be observed best. Pictures are taken immediately.

2.8.2 Flat preparations

For detailed analysis of the ventral midline and the NE spider embryos are flat prepped. Single embryos are transferred from the cap to a microscope slide in a drop of either 70% glycerol in PBS (visible stainings) or a 1:1 mixture with Vectashield mounting medium (fluorescent stainings). The legs that obscure the NE are removed and the embryo is opened up from the dorsal side with fine needles. The yolk is removed so that both the midline and NE are free from any hindering material. The preparations can then be covered carefully with a cover slip and the edges are sealed with nail varnish. All slides are stored at -20°C until documentation.

2.8.3 Documentation of antibody stainings

Pictures of whole mount embryos with visible staining are taken with a stereo microscope, equipped with a camera. Flat preparations with fluorescent as well as double stainings that combine *in situ* and fluorescent stainings are documented with a confocal laser scanning microscope from the companies Leica and Zeiss (LSM, TCS SP2 in Mainz; LSM5 in London). The LSM is capable to detect both visible and fluorescent staining at once.

Solitary visible stainings are recorded with light microscopes from Zeiss or Leica.

2.8.4 Evaluation and analysis

Evaluation and the first processing of the raw confocal data are done with the according software versions LCS Lite from Leica and the ZEN light edition from Zeiss. For visible stainings the Zeiss Axiovision and the Leica Application Suite are used.

Adobe Photoshop and Illustrator are used to edit the processed confocal data and pictures of the visible stainings and to put these together into picture panels.

3 Results

3.1 Development of the embryonic axon scaffold in the spiders *Cupiennius salei* and *Achaearanea tepidariorum*

The organisation of the spider nervous system has been described and morphologically characterised in adult spiders to some extend. The question of how the neuropile and the axonal scaffold are established during embryonic development has remained elusive to this date and shall be answered in the following. To analyse the formation of the segmental neuromeres, follow the process of axon outgrowth and scaffolding of the ventral nerve cord (VNC) it is vital to visualise contributing neural precursor cells, differentiating neurons and forming axonal projections. The antibodies against *At* Cadherin (Cadherin) and acetylated α Tubulin (Tubulin) provide sufficient tools to follow axonogenesis throughout embryonic development.

As described before, the CNS in chelicerates arises from the ventral neurogenic region (Anderson, 1973). Through proliferation a large number of neural precursor cells is generated, which invaginate to form the invagination groups (NPGs) that are arranged in a stereotypic pattern. Eventually in *Cupiennius* 38 of these primary NPGs are formed, *Achaearanea* totals a number of 37 invagination sites per hemisegment (Döffinger and Stollewerk, 2010; Stollewerk et al., 2001). The neural precursor cells then differentiate and are incorporated into the neuromere and start to form axonal projections to establish the VNC.

3.1.1 Axonogenesis in *Cupiennius salei*

The first expression of *At* Cadherin in spiders can be seen early in the embryo, before the germ band is beginning to form (Oda et al., 2005). When the germ band elongates and the NE is defined in *Cupiennius*, Cadherin is expressed in all neuroectodermal cells, but particularly visible in the invagination sites. The cells of

the neural precursor groups are arranged in epithelial vesicles and show a bottle-shape morphology with long extensions in the apical layer of the NE that are tightly packed. Hence the invagination groups are visible as dots within the segment in horizontal optical sections of embryos stained for anti-Cadherin and anti-Tubulin respectively. At first the segmental neuromeres in the prosoma become visible. In the cheliceral, the pedipalpal and the four leg bearing segments the individual ganglia are formed synchronously, while in the opisthosoma the neuromere is not yet recognisable (Fig. 3-1 A). Opisthosomal thickenings are formed later and follow the developmental gradient from anterior to posterior with anterior segments being further advanced than posterior ones. The neural precursors of the invagination groups delaminate from the vesicles and differentiate into neural cells. The first differentiating cells originate from centrally located invagination groups and are positioned medial in the hemisegment (Fig. 3-1 A, E, I), their projections are directed towards the centre of the segment (Fig. 3-1 I). With the beginning of axonogenesis at around 160h of development, Cadherin expression can be seen in the forming neuropile (Fig. 3-1 A, E). Antibody stainings against both Cadherin and Tubulin reveal that differentiation and fasciculation already take place, while NPGs are still visible in the apical layers of the NE (Fig. 3-2 A-C and D-G). In the forming neuromeres groups of cells can be identified in medio-basal sections, which have delaminated into the embryo and develop axonal projections. The growth cones are orientated towards the segmental centre (Fig. 3-2 F). Newly formed axons extend from medial to basal layers of the NE, where they participate to establish the embryonic axon scaffold (Fig. 3-2 D, H). The longitudinal scaffold of the embryonic nervous system becomes visible, when axon fascicles join the neuropile of adjacent hemisegments at about 210h of development. At the same time the first commissural axons cross the midline.

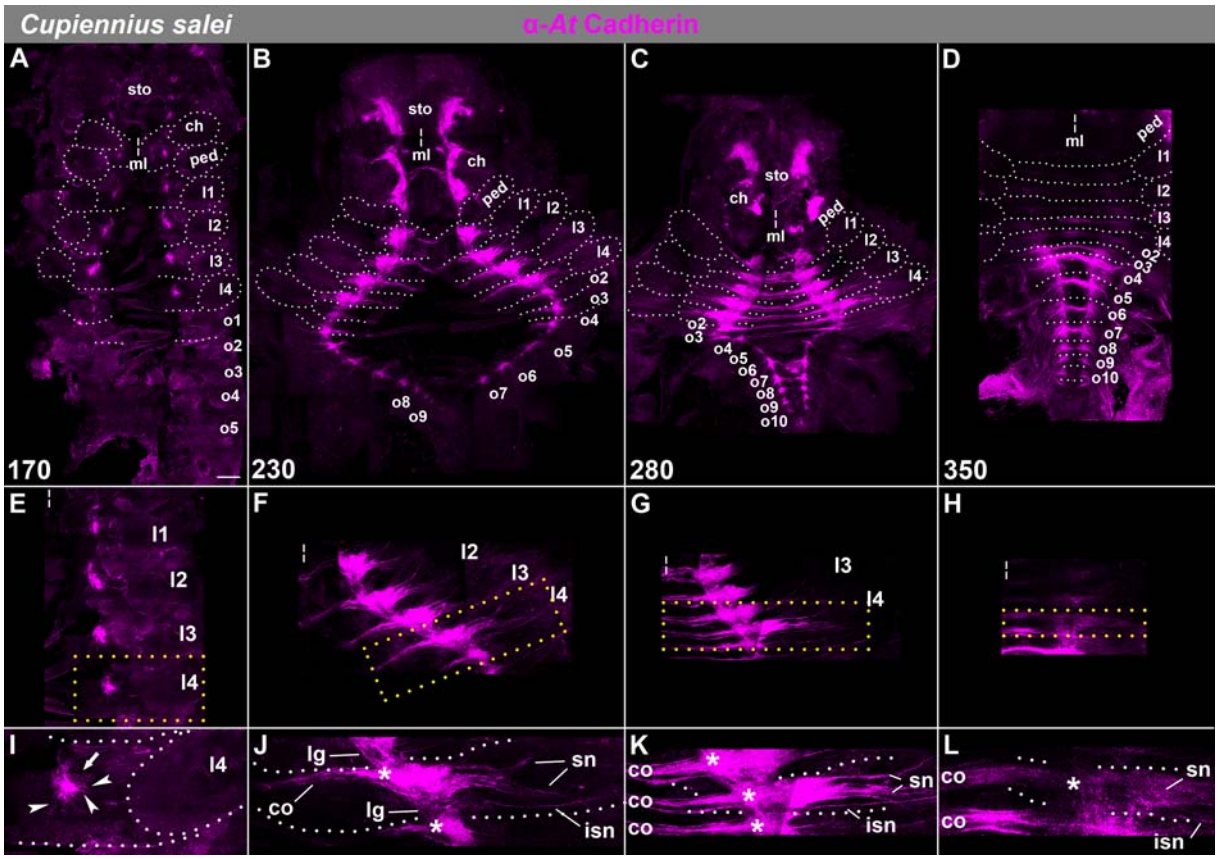

Figure 3-1: Development of the embryonic axon scaffold in *Cupiennius salei* during embryogenesis.

Antibody staining against *At* Cadherin in *Cupiennius salei* embryos at different developmental stages to illustrate the establishment of the embryonic axon scaffold. The panels show maximum projections of basal confocal layers of complete flat preparations (**A**-**D**), close-ups of the leg hemisegments (**E**-**H**) and magnifications of one single hemineuromere (**I**-**L**). (**A**) Flat preparation of an embryo at 170h of development. Clearly visible are the forming neuromeres in prosomal segments. (**E**) Magnification of the leg bearing segments. (**I**) Higher magnification of the individual leg segment 4 (yellow dotted square). Arrow heads point towards axonal projections that come from the first differentiated neurons and grow towards the segmental centre. (**B**) Flat preparation of a *Cupiennius* embryo during mid-inversion. The ventral epithelium, that covers the width between the two germ band halves has almost reached its broadest expansion. The embryonic axon scaffold is well developed. The segmental neuropiles of each embryonic half are interconnected longitudinally. The germ band halves are connected by one commissure per segment. Still obvious is the anterior-posterior gradient of maturation. (**F**) The architecture of the scaffold is best seen in the leg segments. (**J**) Magnification of one leg segment reveals that per hemisegment one commissure exits the NE and grows towards medial to cross the midline. The commissural fibres leave the neuropile at the anterior most position (asterisk) adjacent to the segment boundary (dotted line) in a singe fascicle. The longitudinal tracts are rather short. The periphery is innervated by a segmental nerve that leaves the neuropile close to the anterior segment boundary opposite from the commissural tract. Halfway between the neuropile and the lateral border of the NE the fascicle bifurcates before innervating the legs. The intersegmental nerve exits the CNS at the posterior border of the neuromeres. (**C**) After dorsal closure the midline epithelium becomes reduced again and the embryo becomes more compact in general. All of the segments of the opisthosoma have formed. (**G**) Higher magnification of the leg bearing segments. (**K**) At the level of a single hemisegment one can observe the condensation of the longitudinal tracts to a degree where they are hardly recognisable between the neuromeres. The commissures still consist of single fascicles and are positioned in close proximity. The segmental nerve (sn) and the intersegmental nerve (isn) are well distinguished, the intersegmental nerve lies closer to the segmental nerve than in the previous stage. (**D**) At the end of embryogenesis the axon scaffold shows the typical characteristics of an arthropod CNS. Especially in the opisthosoma the rope-ladder like structure becomes evident.
Legend continues next page (44).

Legend to Figure 3-1: (**H**) Close-up of leg bearing segments (**L**) Right to the end of embryonic development only a single commissure crosses the midline although this has become quite thick due to the reduction of the midline epithelium. **ch**; chelicera, **co**; commissure, **isn**; intersegmental nerve, **l1-l4**; walking leg 1-4, **lg**; longitudinal, **ml** and dashed line; midline, **o1**-**o10**; opisthosomal segment 1-10, **ped**; pedipalp, **sn**; segmental nerve, the scale bar is 100µm.

Spiders undergo tremendous morphological changes in a morphogenetic process called inversion, which starts in *Cupiennius* at around 180-190h of development. During this process the germ band halves separate ventro-medially and move towards lateral and dorsal. The ventral midline epithelium is continuously expanded along the way (Seitz, 1966; Foelix, 1996).

During mid-inversion in the Cadherin staining the longitudinal fascicles appear to be rather short so that neighbouring neuromeres, especially in the prosoma, lie immediately adjacent to and almost touch each other. In the opisthosoma they are further apart (Fig. 3-1 B). The neuropile thickens as more axonal projections fasciculate with the initial pioneering tracts. The anterior-posterior gradient of nervous system development becomes apparent in that the neuromeres in the prosomal segments and the anterior opisthosomal segments are further developed than the ones of the very posterior segments, where they are still relatively small and commissures are only beginning to form (Fig. 3-1 B). The largest neuromeres formed are the ones of chelicerae, the pedipalps and in the segments of the walking legs (Fig. 3-1 F; Fig. 3-2 J). Before leaving the NE to cross the ventral midline, commissural axons grow alongside the intersegmental boundary. They exit the NE in a single nerve bundle at the anterior most corner of the hemisegment (Fig. 3-1 J; Fig. 3-2 M). During inversion, the commissural tracts follow the movement of the two germ band halves. They expand dorso-ventrally and eventually span the entire width between both embryo halves. Perpendicular to the longitudinal axis of the embryo, two axon tracts leave the neuropile and grow towards the periphery (Fig. 3-1 F; Fig. 3-2 J). I termed them according to *Drosophila*. The segmental nerve (SN) grows straight from the neuromere in the direction of the appendages, opposite to where the commissure leaves the NE. The nerve bundles of the pedipalps and the leg bearing segments are thickest and bifurcate before they apparently innervate the appendages (Fig. 3-1 F, J; Fig. 3-2 J, M). The intersegmental nerve (ISN) exits the CNS in between two adjacent hemisegments and continues towards the periphery in one fascicle (Fig. 3-1 J; Fig. 3-2 M). Double staining with anti-Tubulin and anti-

Cadherin show the projections within the neuropile and the commissures. Axonal projections seem to explore their surroundings, some of them joining the commissural tracts, some of them contributing to the peripheral nerves, while others add to the longitudinal axon scaffold (Fig. 3-2 I, K, L, N). The formation of the cuticle towards the end of embryogenesis hampered the analysis of later developmental events using anti-Tubulin.

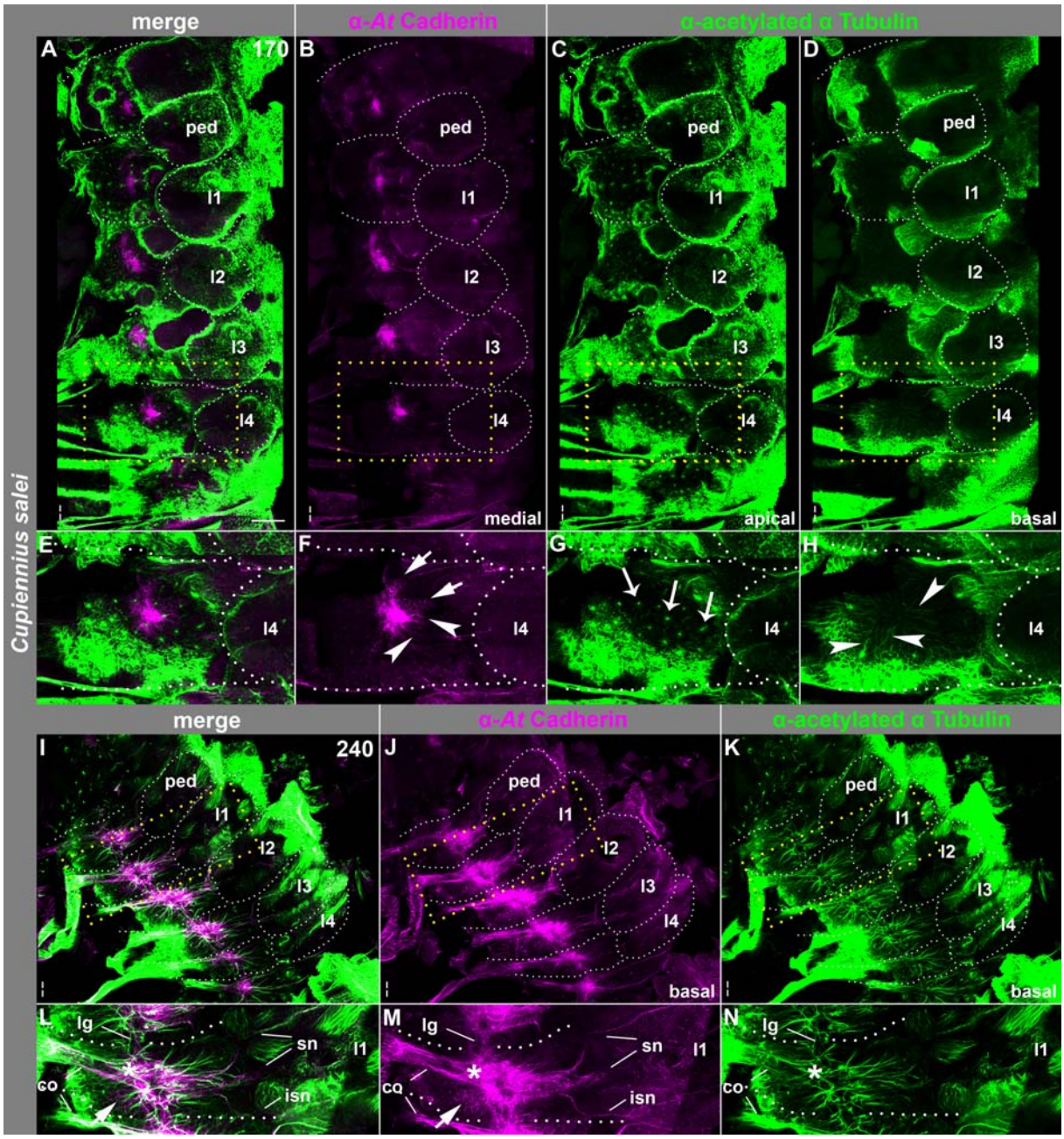

Figure 3-2: Double staining against anti-At Cadherin and anti-acetylated α Tubulin reveals more details of embryonic scaffolding.

Legend see next page (46).

Legend to Figure 3-2: The panel shows double stainings of flat preparations of *Cupiennius* embryos against *At* Cadherin (magenta) and acetylated α Tubulin (green). Confocal stacks of prosomal segments of a *Cupiennius* embryo at 170h of development (**A**-**D**). Magnification of one leg hemisegment of the embryo (**E**-**H)**. *Cupiennius* embryo at 240h during mid-inversion (**I**-**K**), shown are the leg hemisegments. Details of a single hemisegment (**L**-**N**). (**A**) and (**E**) show an overlay of both antibodies in confocal sections of apical and medial layers. The close-up of a single segment demonstrates how cells have left the compound of the invagination sites to differentiate and add to the forming neuropile, while apically NPGs still persist. (**B**) and (**F**) show a Cadherin staining of the medial layers. One can clearly see the neuropile is being established. In the magnification in (**F**) arrowheads indicate axonal projections that have formed, their growth is directed towards centrally. Arrows point towards cells that have invaginated to differentiate and built up the neuromere. (**C**) In apical layers of the NE the invagination sites are marked by anti-Tubulin. (**G**) Arrows indicate the position of three of them in the magnification of one leg hemineuromere. (**D**) In the more basal layers outgrowing axonal projections express Tubulin. (**H**) In the close-up of one hemisegment they are marked by arrowheads. (**I**) In the overlay of Cadherin and Tubulin the neuromeres of prosomal hemisegments are seen. (**L**) The magnification of one hemineuromere illustrates the integration of axonal projections during nervous system scaffolding. (**J**) and (**M**) show the Cadherin only. (**M**) Organisation of one hemineuromere in the close-up. The longitudinals connect neighbouring neuromeres to form a continuous VNC. Commissural axons grow out to cross the midline. The asterisk marks the point, where they leave the NE. At the lateral side of the hemisegment sn and isn can be recognised as they grow towards the periphery. The arrow points at differentiated cells contributing to the neuronal mass of the neuromere (**K**) The Tubulin staining alone resolves the interlacing of axonal projections that make up the nerve cord. (**N**) It becomes particularly obvious that the longitudinal and commissural tracts are made up by several projections that fasciculate with each other. However the great number of axons marked by Tubulin makes it difficult to interpret the staining. **co**; commissure **dashed line**; midline, **isn**; intersegmental nerve, **l1-4**; leg hemisegments 1-4, **lg**; longitudinal, **ped**; pedipalp, **sc**; segmental commissure, **sn**; segmental nerve, scale bar is 100µm.

Axonal tracts in *Cupiennius* are presumably pioneered by segmentally organised cells as double staining of Phalloidin and the neural cell fate determinant Prospero show (Fig. 3-3). Clearly visible in these stainings at around 250h are the distribution of invagination sites persisting in apical layers, while in medial regions of the NE differentiating neural cells marked by a *Cs* Prospero antibody are found. The forming neuropile is visible basally.

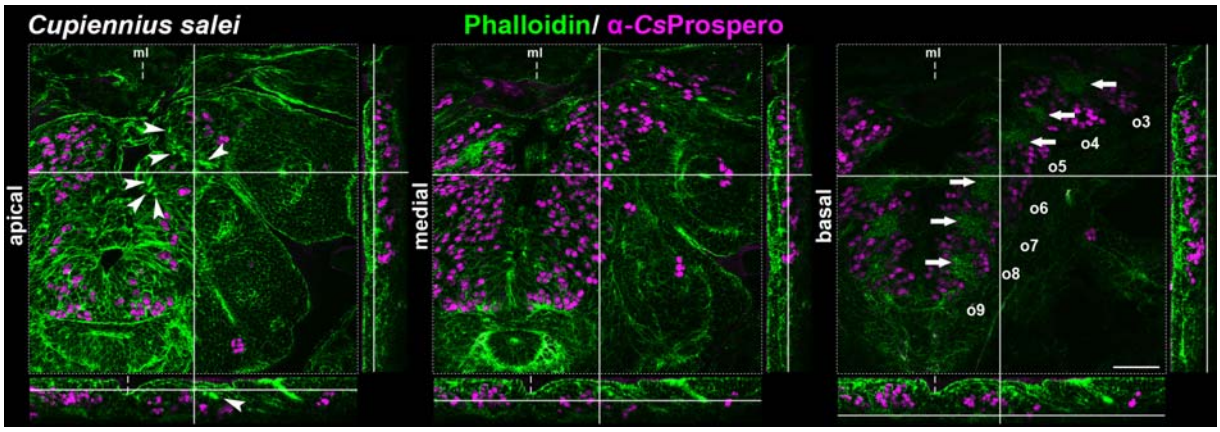

Figure 3-3: Distribution of NPGs, differentiating neural cells and axon scaffold within the NE of *Cupiennius salei* from apical to basal layers of confocal sections.

The panel shows Phalloidin staining (green) combined with an antibody against *Cs* Prospero (magenta) on a *Cupiennius* embryo of around 250h. The embryo had been prepared and the last opisthosomal segments are shown. Arrowheads point to the NPGs that still persist apically in the NE. The differentiating neural cells that delaminate from the invagination groups and migrate towards more medial positions of the NE show expression of Prospero. The neuropile established by differentiated neurons can be found in the basal most layers of the NE. Individual neuromeres of one hemisegment are indicated by arrows. **ml** indicates the position of the ventral midline, **o3-9**; opisthosomal segment 3-9, scale bar is 80µm.

After dorsal closure at around 260h when the two germ band halves have meet dorsally the midline becomes reduced again (Fig 3-1 C). The embryo has become even more condensed, which can be seen in the prosoma, where the individual neuromeres move closer together so that the longitudinal tracts between them can no longer be distinguished (Fig. 3-1 C, G, K). Also in the abdomen the neuropile is more compact than before (Fig. 3-1 C). Due to this condensation of the entire neuropile the commissures come to lie in close proximity (Fig. 3-1 G, K).

Inversion ends at the end of embryogenesis when the ventral midline is completely reduced and the opposite halves of the germ band meet again ventrally. The embryo bends around a transverse furrow and is morphologically clearly separated into prosoma and opisthosoma (Seitz, 1966; Foelix, 1996). The ventral nerve cord of *Cupiennius* has adopted the typical appearance of an arthropod nervous system (Fig. 3-1 D). Due to condensation of the VNC the neuropile increasingly resembles the nervous system of the adult spider, where all opisthosomal neuromeres are condensed into the compact abdominal nerve mass. Nevertheless and importantly during the whole of embryogenesis all of the segments show individual neuromeres that can be readily distinguished. The complete condensation of the abdominal nerve mass with the prosomal neuropile does not occur during embryonic stages. The

separation of the commissural tracts into several bundles as seen in the adult must take place during larval stages since I only found one commissure crossing the midline in each segment. Using *At* Cadherin as a tool, I could show that the embryonic nervous system of *Cupiennius salei* in the prosoma consists of the cephalic neuropile and the six separate neuromeres of the chelicerae, the pedipalps and of the four walking legs. In the abdomen I was able to identify nine to ten individual neuromeres, o1-o10, depending on the developmental stage. All hemineuromeres are interconnected by one commissure per segment.

3.1.2 Axon scaffolding in *Achaearanea tepidariorum*

In general the course of events taking place during embryonic nervous system development is almost identical in the two spider species analysed. However, as stated initially, embryonic development of *Cupiennius* takes about twice as long as in *Achaearanea*. The developmental stages given refer to observations made by Dr. Beate Mittmann, who is currently preparing a detailed staging in *Achaearanea*. In *Achaearanea* I analysed developmental stages that correspond to the ones described in *Cupiennius*. Similar to *Cupiennius* in the embryo of *Achaearanea* the prosomal segments develop simultaneously. The segmented germ band then elongates by adding opisthosomal segments in the anterior to posterior fashion described before. At the same time the limb buds appear. The first specific expression of Cadherin in the NE can be seen in *Achaearanea* at the beginning of neurogenesis at around 75h (corresponds to 130h in *Cupiennius*) when the first NPGs appear. As described before in confocal sections these are visible as dots in the apical layers of the NE. The number and arrangement of the NPGs is strikingly similar to *Cupiennius*. 37 individual NPGs were identified per hemisegment (Fig. 3-4 A, E, I) (Döffinger and Stollewerk, 2010). With the start of differentiation in the first neural cells, Cadherin expression can be seen in neurons and their axonal projections that are the foundation of the developing axon scaffold (Fig. 3-4 B, F, J). Again the neuropile is formed basally while invagination groups persist and the axonal scaffold in the prosoma appears simultaneously and strongly expresses Cadherin.

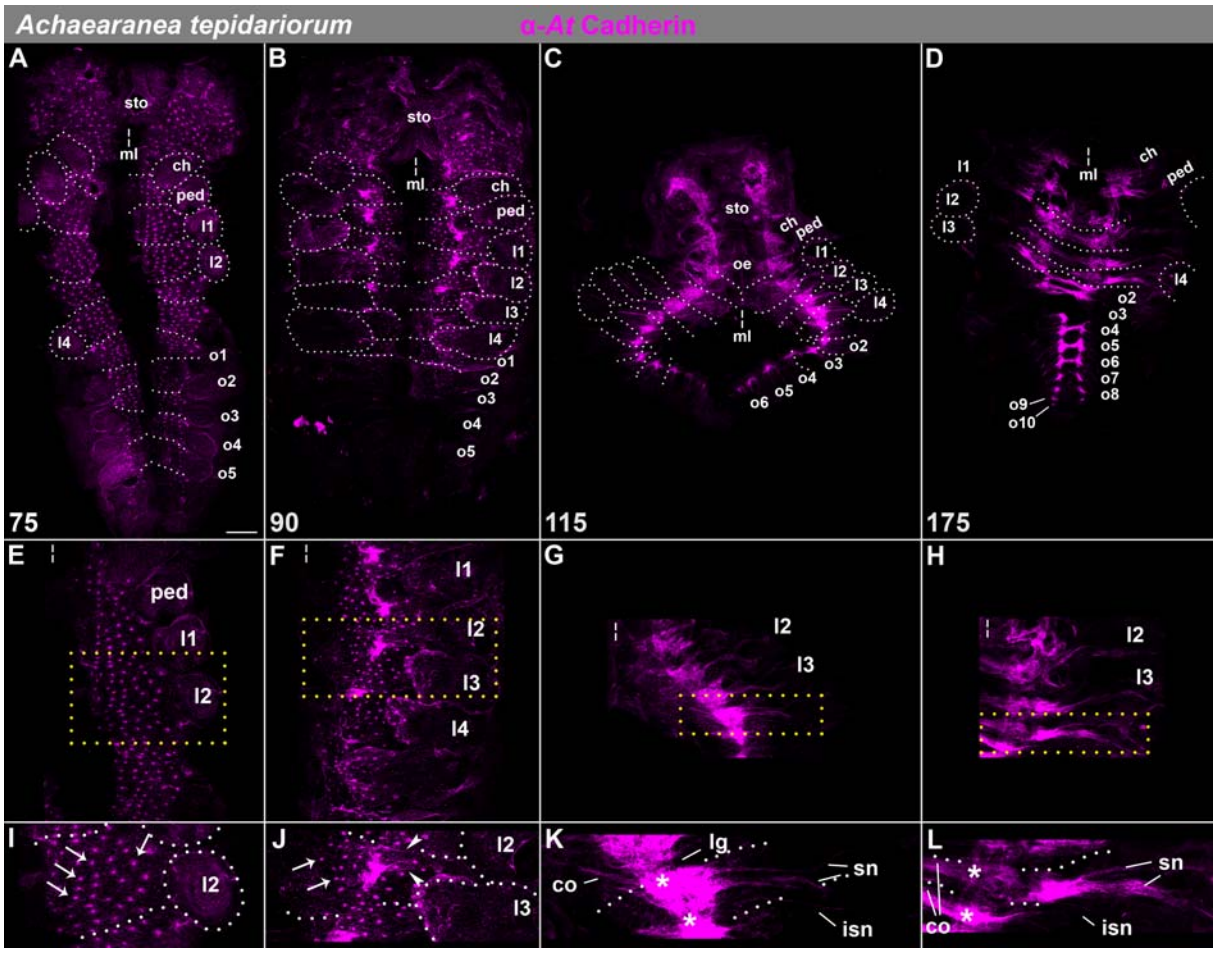

Figure 3-4: Axon scaffolding during embryonic development of *Achaearanea tepidariorum*.

Flat preparations of *Achaearanea* embryos of successive developmental stages stained against the *At* Cadherin antibody. Confocal micrographs of preparations of complete embryos illustrate the morphological changes going on during embryogenesis (**A**-**D**). Higher magnifications of hemisegments of the four walking legs (**E-H**). Details of one individual leg hemineuromere (**I**-**L**). (**A**) Maximum projection of apical confocal sections. During early neurogenesis at around 75h the PNGs are formed and are visible as dots in the apical layers of the NE. Prosomal segments are developmentally further advanced than segments of the opisthosoma. (**E**) Close-up of one leg hemisegment, arrows mark individual invagination sites. (**B**) Maximum projection of apical to medial layers of confocal stacks. At the onset of inversion at 90h the ganglia are beginning to form and build up the neuromeres. At the same time PNGs are still visible in the NE. (**J**) In the single hemisegment the NPGs that persist at the beginning of scaffolding are marked by arrows, the arrowheads indicate projections growing out from differentiated neurons to establish the neuropile. (**C**) Basal optical sections in a maximum projection of a flat preparation of an embryo at around 115h. During mid-inversion the embryonic axon scaffold in its basic organisation is almost fully developed. The segmental neuromeres are interconnected by longitudinal tracts to form a continuous neuropile, commissural tracts cross the midline. All of the opisthosomal segments are formed but still lack behind developmentally. (**K**) Commissures have formed and exit (asterisk) the NE to span the midline. Visible are also the segmental and the intersegmental nerve that project towards the periphery. As in *Cupiennius* the sn separates into two fascicles before reaching the legs. (**D**) Maximum projection as described in **C**. After dorsal closure the midline becomes reduced and the commissures shorten along with the reduction. At the end of embryogenesis at around 175h the rope-ladder like structure of the typical arthropod nervous system is evident. (**L**) Close-up of the fourth leg hemisegment. The commissure is very broad but no subdivision into more than one fascicle can be observed. Also the segmental nerve has strongly developed to innervate the legs. Between individual neuromeres the longitudinal tracts are hardly visible anymore. **ch**; chelicera, **ml**; midline, **l1-l4**; leg 1-4, **isn**; intersegmental nerve, **o1-o10**; opisthosomal segment 1-10, **ped**; pedipalp, **sc**; segmental commissure, **sn**, segmental nerve, **sto**; stomodeum, the scale bar is 100 µm.

In parallel to the Cadherin staining I used the antibody against Tubulin to unravel axonogenesis in *Achaearanea*. Tubulin, just like Cadherin, shows the dot like expression pattern in the NE when the NPGs are formed (not shown). A little later strong Tubulin expression can be seen in differentiated neurons and their axonal projections. These neurons are arranged in groups of one to four cells. At around 80h, groups of neurons are located centrally in the cephalic lobe. In the segments of the prosoma the clusters are found in the ventro-lateral region of the NE (Fig. 3-5 A, B). In the ventro-lateral area of the opisthosomal hemisegments I found relatively large cell clusters containing about 7-20 cells. The first of these clusters can be found in the third segment of the abdomen. This one is the smallest of the abdominal clusters but shows axonal outgrowth, which can not be seen in the more posterior positioned larger cell groups. This observation could be explained by the gradient of developmental maturation along the anterior-posterior axis. Thus in the third abdominal segment Tubulin expression has already dwindled in some of the cell somas of the clustered neurons as they have developed axons. In more posterior segments this is not yet the case (Fig. 3-5 C). Individual invagination groups are still present in all segments. Interestingly I do not find these clusters of pioneer neurons in *Cupiennius* at any stage analysed. Shortly after that, at around 95h in each hemisegment of the prosoma a large number of Tubulin positive cells with outgrowing projections are located centrally, while in the abdomen the lateral clusters are still visible (Fig. 3-5 D). The axons of several of these neurons fasciculate and their cell bodies lie close together. Most axonal projections formed by these cells point towards the centre of the segment. At the anterior-medial most area close to the segment boundary, exactly the position where commissures begin to grow out from the NE, a number of neurons are located. Their axonal outgrowth is directed towards the neuromere, parallel to the segment border (Fig. 3-5 E). Apart from the neuronal clusters in the opisthosoma, which show now slightly reduced Tubulin expression, one cell is located anterior-medial in the hemisegment that shows a very pronounced axonal projection growing towards the periphery. At the beginning its course is directed towards the segment boundary before growing alongside it (Fig. 3-5 F).

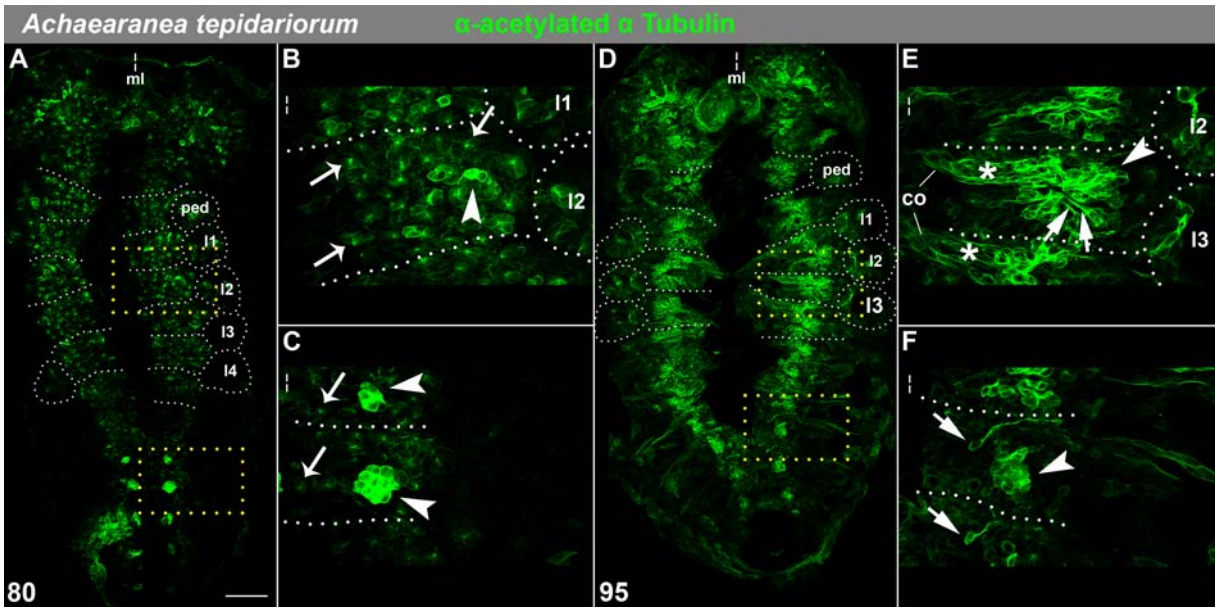

Figure 3-5: Axonal scaffolding during embryonic development of *Achaearanea tepidariorum*.

The panel shows confocal micrographs of flat preparations of *Achaearanea* embryos at different developmental stages stained with the antibody against acetylated alpha Tubulin. Overview of preparations of the entire embryo (**A**, **D**). Higher magnification of one of the prosomal hemisegments (**B**, **E**). Magnification of an opisthosomal hemisegment (**C**, **F**). (**A**) Maximum projection of apical layers. At 80h predominantly in prosomal segments cell clusters that are strongly Tubulin positive are located in the lateral part of the hemisegment. Groups of neurons can be seen in the cephalic lobes as well. In the opisthosoma large clusters were found that strongly express Tubulin and contain up to 20 cells. (**B**) The prosomal clusters consist of one to four cells (arrowhead), which are probably differentiated pioneer neurons. Axonal outgrowth is already visible. Apart from that, NPGs are still evident (arrows) in the higher magnification of a leg hemisegment. (**C**) In opisthosomal segments one large cluster, containing 7-20 cells depending on their position along the anterior-posterior axis, can be found. (**D**) Maximum projection of medial confocal layers. At 95h the centre in all prosomal and some opisthosomal segments is occupied by strongly Tubulin expressing neurons. Some are also present in the head. (**E**) Close-up of a leg hemisegment. The differentiated neurons (arrowhead) show axonal projections, which are directed towards the centre of the hemisegment and often fasciculate with each other (arrows). Additionally there are cells visible that populate the anterior-medial most region of the NE (asterisk) and possibly correspond to the commissural pioneer neurons. (**F**) In the opisthosomal hemisegment expression of Tubulin in the cell clusters observed earlier in development is less strong (arrowhead). Furthermore there is an additional cell present per hemisegment, which is positioned anteriorly to the large cluster within the hemisegment and is strongly Tubulin positive (arrow). This single cell develops a very pronounced axon, which grows towards the periphery along the segmental border. **ch**; chelicera, **ml**; midline, **l1-l4**; leg 1-4, **o1-o10**; opisthosomal segment 1-10, **isn**; intersegmental nerve, **ped**; pedipalp, **sc**; segmental commissure, **sn**, segmental nerve, **sto**; stomodeum, the scale bar is 100µm.

During inversion fasciculation of the embryonic nervous system continues. Longitudinal tracts connect the neuromeres in anterior to posterior direction and commissural tracts start to grow out from the NE to connect both germ band halves. On the lateral side of the neuromere axon bundles are beginning to innervate the periphery (Fig. 3-4 C). As in *Cupiennius*, in *Achaearanea* I identified a segmental and an intersegmental nerve. The SN also shows a division into two separate branches in prosomal segments (Fig. 3-4 G, K). Opisthosomal segments clearly show the anterior

to posterior gradient of maturation where the more anterior lying segments are further advanced than the ones more posterior. In developmentally younger segments the neuromeres are smaller and the contralateral and longitudinal connections between them are less defined (Fig. 3-4 C).

After dorsal closure the ventral midline becomes restricted again and the two germ band halves meet ventrally, inversion is complete at around 180h. With the reduction of the ventral midline the commissures shorten again, so that at the end of embryogenesis the VNC in spiders shows the typical arthropod rope-ladder like structure (Fig. 3-4 D). The condensation of the CNS as seen in the adult spider becomes evident in the late embryo. As is the case for *Cupiennius* also in *Achaearanea* the two halves of the germ band are interconnected by only one commissure per segment throughout embryonic development. To the end of embryogenesis individual neuromeres can be distinguished. Taken together, I found that the neuropile of the embryo in both species comprises of the same number of prosomal (6) and opisthosomal (9-10) neuromeres and commissures that where visualised using the antibodies against Cadherin and Tubulin. The development of the embryonic neuropile is similar in both *Cupiennius* and *Achaearanea* with respect to the events taking place as well as the final outcome.

3.2 The spider ventral midline

The midline is a critical organising centre for the developing CNS in bilaterally symmetrical organisms and plays a major role in the establishment of axonal tracts as well as being the source of guidance molecules that are involved in axon path finding.

In spiders the ventral midline is an epithelium that separates the two germ band halves ventro-medially. It was termed ventral sulcus by Anderson (1973). During inversion it expands as the germ band halves withdraw from each other and reaches its widest expansion when the embryo has closed dorsally and is beginning to bend around a transverse furrow between prosoma and opisthosoma. After dorsal closure when both germ band halves start approaching each other again, this epithelium

becomes reduced again and is extinct at ventral closure at the end of embryogenesis. The midline area is also devoid of any neural cells as shown by analysis of different markers (Döffinger and Stollewerk, 2010; Stollewerk et al., 2001) and it could be comparable to the vertebrate floor plate. Nevertheless, the ventral median area in *Achaearanea* expresses *single minded* (*sim*) (Akiyama-Oda and Oda, 2005), which in *Drosophila* functions as the key regulatory gene of midline cell specification.

One aim of this thesis was to analyse the ventral midline in spiders and its potential role in embryonic development and axon pathfinding. In the following section a detailed analysis of *sim* expression in *Achaearanea* is given. Furthermore I attempted to down regulate *sim* in the embryo using embryonic RNA interference.

3.2.1 The expression pattern of *single minded* in *Achaearanea* shows both *Drosophila* and vertebrate features

Spider *sim* was originally cloned by Akiyama-Oda and Oda (2006) in *Achaearanea tepidariorum*. I generated specific primer based on the published sequence and reproduced an identical product (for sequences see appendix). The expression pattern of *sim* transcript was analysed by using whole mount *in situ* hybridisation with a DIG-labelled *At sim* RNA antisense probe generated.

In *Achaearanea sim* is expressed in the ventro-medial area of the embryo, at first in a broad median domain along the ventral side (Fig. 3-6 A; Fig. 3-7 A-D). This expression comes up at around 75h, as soon as the germ band has elongated to its final length and the prosomal limb buds are beginning to form as shown before (Akiyama-Oda and Oda, 2006). This is before the two halves of the NE are separated by a median furrow and before the onset of inversion. Expression of *sim* is broadest at the segmental boundary between adjacent neuromeres (Fig. 3-6 A, E, I). Morphologically cells of the *sim* expressing area can not be distinguished from cells of the NE immediately adjacent to the *sim* domain at this stage of development. Apart from the expression of *sim* transcript in the NE, early on strong expression can be seen at the onset of inversion in the walking leg segments 1-4 and also in the first

opisthosomal limb bud (o2) (Fig. 3-6 B). The *sim* positive cluster is found centrally at the base of the appendage from 80h onwards. This is consistent with findings in both *Drosophila* and vertebrates, where *sim* is expressed in non neurogenic regions such as muscles.

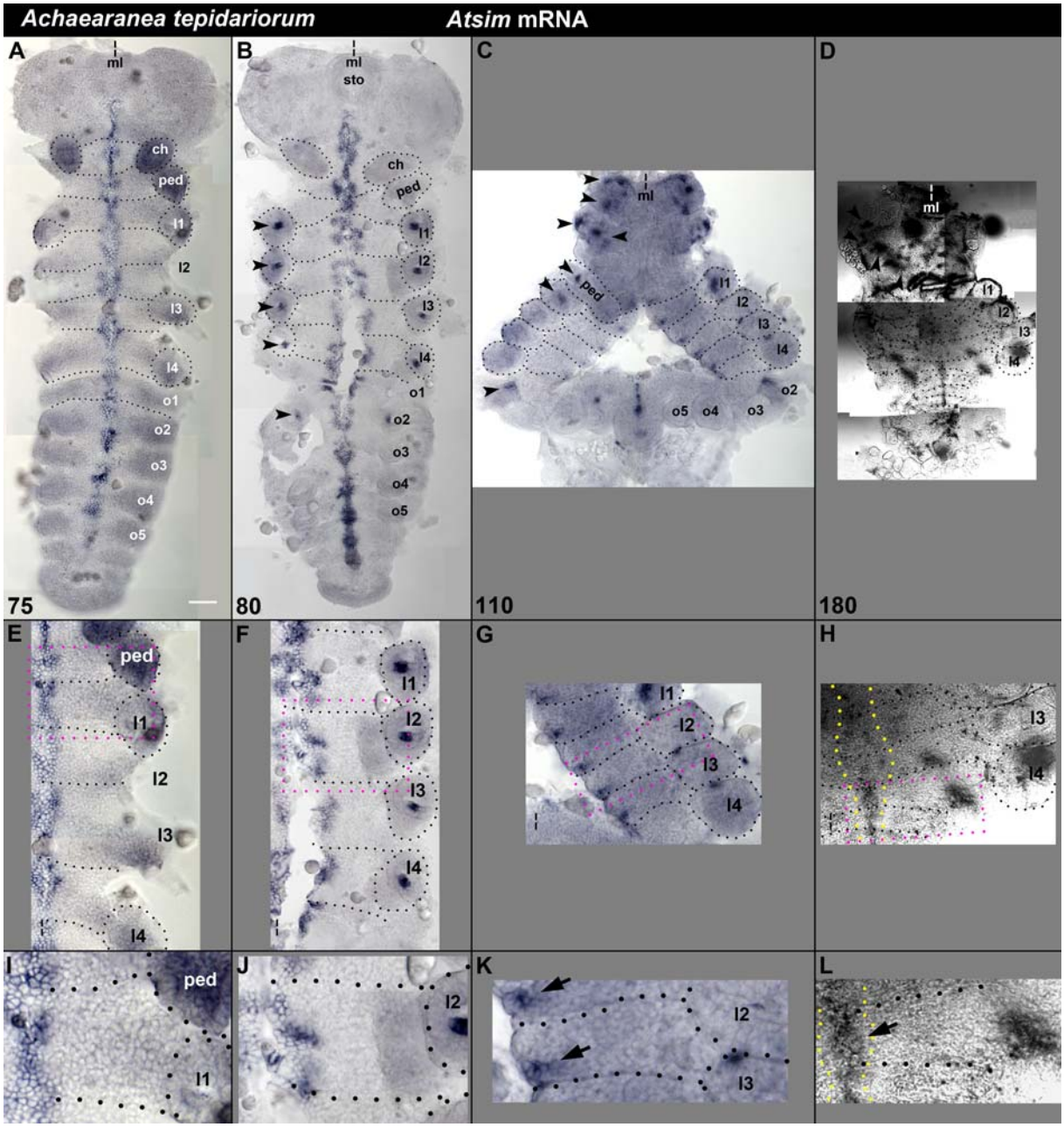

Figure 3-6: Expression of the *single minded* transcript throughout subsequent developmental stages in *Achaearanea tepidariorum*.

In situ hybridisation in *At* embryos of different stages with DIG-labelled *Achaearanea single minded* RNA antisense probe. **A**, **B**, **C**, **D** show flat preparations of complete embryos. **E**, **F**, **G**, **H** Higher magnifications of the leg bearing segments in the right germ band half. **I**, **J**, **K**, **L** Close-up of one individual hemisegment. (**A**) Expression of *sim* comes up at around 75h, when the germ band is fully extended. The transcript can be seen in a single longitudinal stripe along the ventral median side of the embryo.
Legend continues on the next page (55).

Legend to figure 3-6: (**E**) Detail of a single hemisegment. Expression of *sim* is broadest where the segmental boundary (dotted line) constricts the width of the NE. Morphologically the *sim* expressing cells do not look any different from the adjacent cells of the actual NE, which are *sim* negative. (**B**) With the beginning separation of the germ band halves through inversion at around 80h the *sim* domain is split in two. Only at the anterior and posterior ends, where the germ band stays connected throughout the morphological movements, the single stripe of *sim* expression persists. In addition to the expression of *sim* in the NE, *sim* expressing cells located at the base of the legs and the appendage of the second opisthosomal segment were found (arrowheads). (**I**) Expression of *sim* within a single hemisegment is less continuous compared to the early stage. A concentration into clusters of strongly *sim* positive cells medially in the segment can be observed. (**C**) During mid-inversion at around 110h, expression of *sim* has been reduced to cell clusters posterior within segments right next to the single layered tissue that spans the gap between the germ band halves. The *sim* positive cells in the appendages are still visible in the pedipalp, the first leg and the opisthosoma. In the head *sim* expressing clusters can be seen (arrows). (**K**) In the higher magnification of an individual hemisegment the *sim* positive cell clusters (arrows) appear to be positioned in the posterior and medial most corner of the hemisegment (**D**) After dorsal and ventral closure right at the end of embryonic development at 180h the ventral midline epithelium has been reduced. Again in prosomal segments cuticle prevented the sim probe from entering the tissue resulting in a weak signal. In the opisthosoma a stripe of *sim* expressing cells was detected. This stripe comprises segments where the domains of both germ band halves appear to have fused where the two sides have met and the last opisthosomal segments that have never separated. (**L**) In the prosoma the expression of *sim* is rather weak if detectable at all (yellow dotted line). Also it is difficult to distinguish between the individual segments. Nevertheless *sim* is expressed at medial positions in one band (arrow). Again at the segmental boundary this band of *sim* cells is constricted. **ch**; chelicera,; **ml**; midline, **l1-l4**; leg 1-4, **o1-o5**; opisthosomal segment 1-5, **ped**; pedipalp, **sto**; stomodeum, scale bar is 100µm

When inversion begins and the germ band is split into separate halves also the expression domain of *sim* is divided. In both parts of the embryo this domain comprises the medial most cells of the germ band and appears to lie immediately adjacent to the NE (Fig. 3-6 B, F, J). Expression of *sim* within the hemisegment looks somewhat shifted as it has retreated from immediately next to the segment border to a more medial area. The midline epithelium that spans the gap between both parts of the germ band does not show any *sim* expression. Since it is difficult to preserve the tissue of the ventral midline area in dissected embryos, I also looked at whole mount embryos hybridised with *sim* RNA probe to investigate whether any *sim* positive cells can be seen in the midline epithelium. The results show that *sim* transcripts are not present in the ventral midline region of *Achaearanea* (Fig. 3-7).

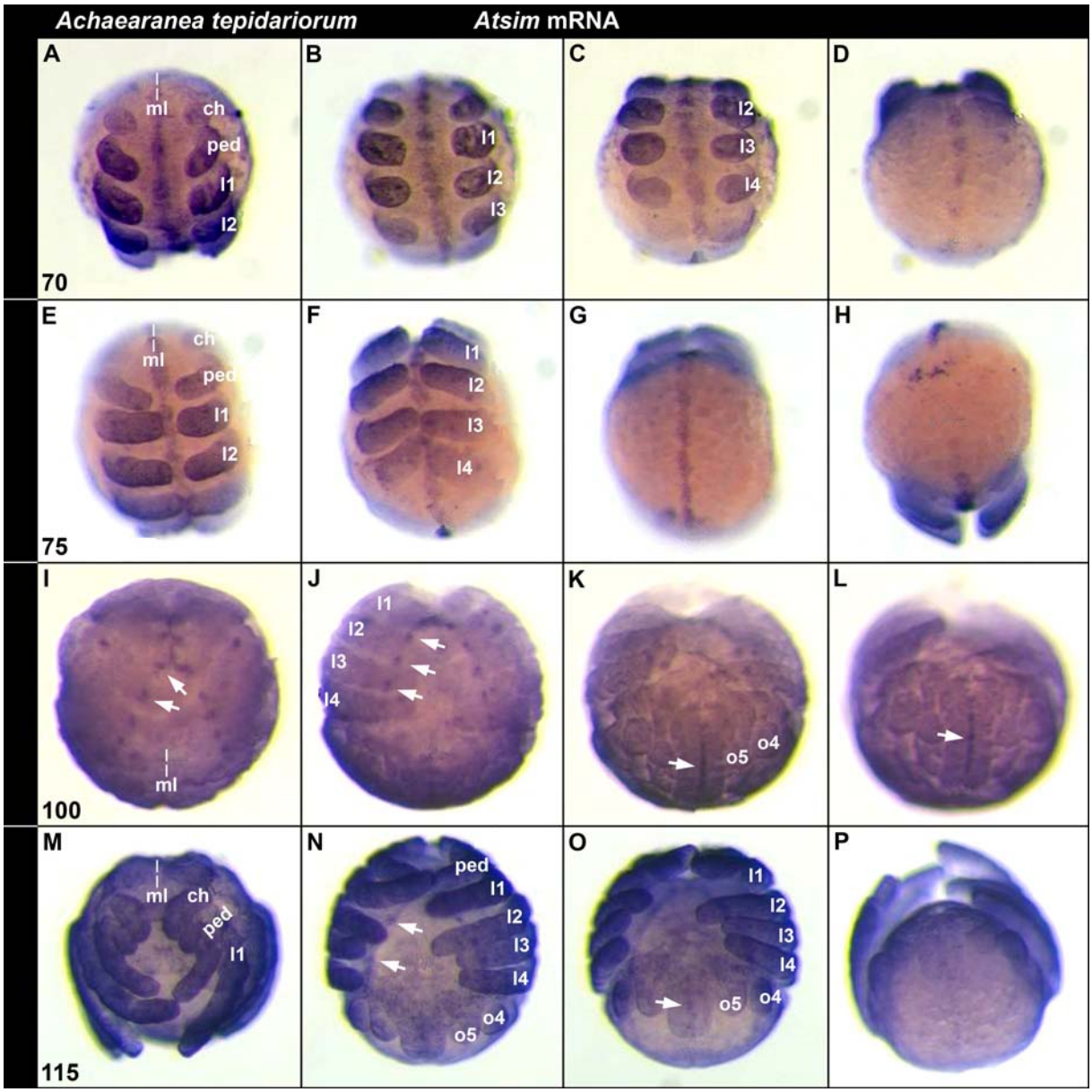

Figure 3-7: Expression of the *single minded* transcript throughout subsequent developmental stages in whole mount embryos of *Achaearanea tepidariorum*.

Whole mount embryos of *Achaearanea* at different developmental stages hybridised with the DIG-labelled antisense RNA probe of *At sim* shown from anterior to posterior. **A**-**D** Embryo at around 70h of development. *Sim* is expressed in a single stripe along the ventral side of the embryo, which is broader at the segmental border and narrower where the hemisegment is widest. **E**-**H** Shortly thereafter, before ventral separation, the legs have elongated but *sim* expression is more or less unchanged. **I**-**L** During mid-inversion *sim* is restricted to clusters of cells in each hemisegment (arrows). Only in the opisthosoma the stripe like pattern is still evident. **M**-**P** After dorsal closure both sides of the embryo start to advance each other again. The *sim* expression pattern nevertheless is the same. **ch**; chelicera, **ml**; midline, **l1-l4**; leg 1-4, **o1-o5**; opisthosomal segment 1-5.

With expansion of the ventral midline epithelium, expression of *sim* becomes restricted to a single cluster of cells in the posterior-medial part of each hemisegment. These clusters can be found in medial to basal layers of the NE, where they are positioned in a pocket like space surrounded by the NE (Fig. 3-8).

Only in the very posterior segments, where both germ band halves stay connected throughout the process of inversion, the stripe like pattern remains intact (Fig. 3-6 C, G, K; 3-7), but also here it is obvious how expression of *sim* has been reduced to a rather narrow band of cells in the segments. The expression of *sim* in the appendages is, except in the pedipalp, the first walking leg and the appendage of the second opisthosomal segment, no longer visible (Fig. 3-6 C). Instead *sim* expression can now be found in various regions of the cephalic lobes, where before no expression of *sim* was detectable (Fig. 3-6 C).

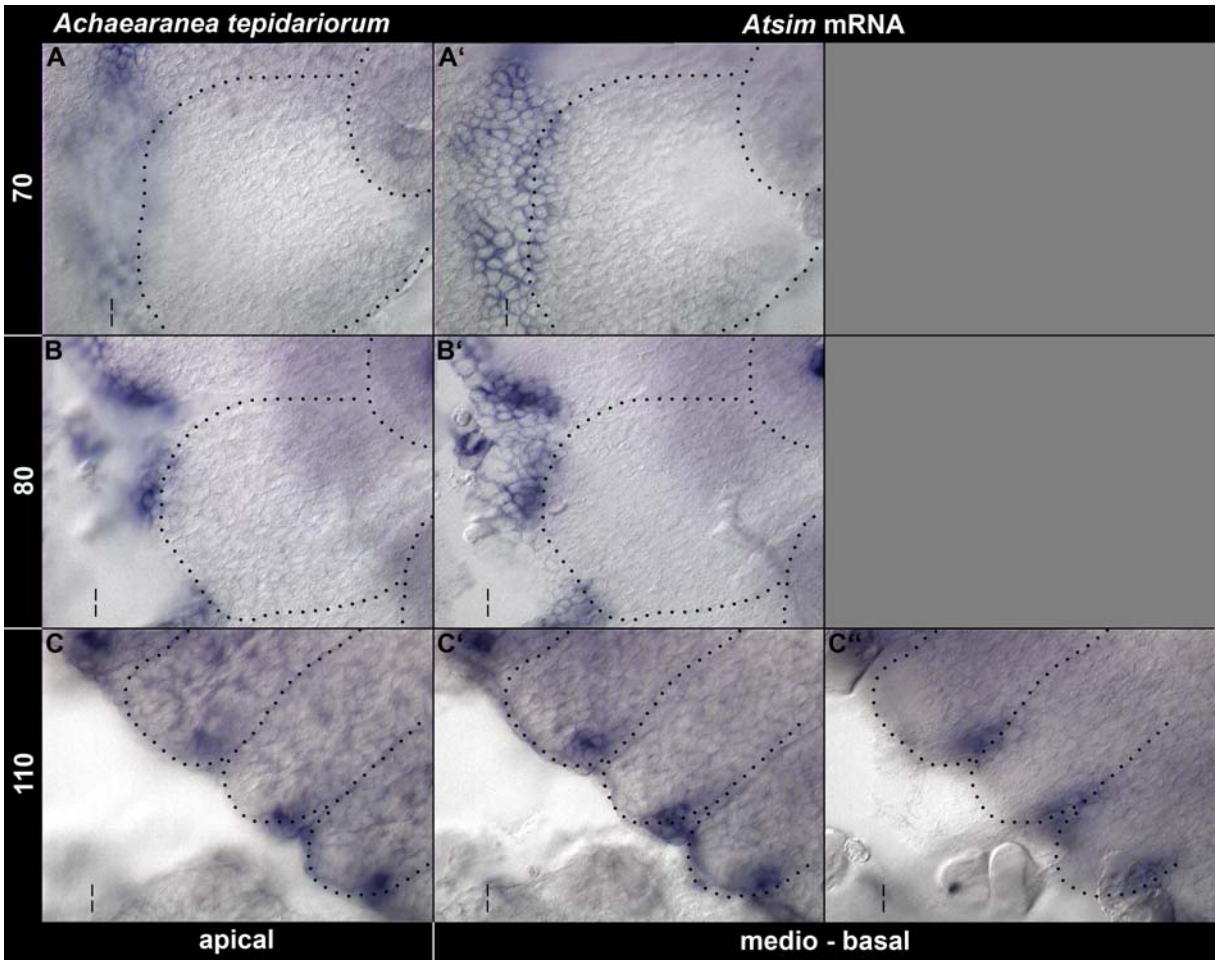

Figure 3-8: *Single minded* is expressed in medial to basal layers of the NE.

Flat preparations of a single hemisegment stained against *sim* RNA. (**A**-**C**) show apical layers of a hemisegment of the NE, (**A'**, **B'**, **C'**, **C''**) Medial to basal layers of the microscopic stacks. The dotted line indicates the outline of neuromere and legs. (**A**, **A'**) During early embryonic stages at 70h *sim* signal is found in medio-basal layers of the NE. At this developmental time point the NE is rather thin. It is obvious that *sim* is not expressed in the neuroectodermal region of the hemisegment but in cells immediately adjacent to it. The expression domain is broadest at the segment boundary. (**B**, **B'**) After the onset of inversion at around 80h of development, expression of *sim* is already restricted to fewer cells rather than a continuous stripe in medial to basal neuroectodermal layers. (**C**, **C'**, **C''**) During mid-inversion at 110h even fewer cells express *sim*. The *sim* positive cells lie at medial-posterior positions of the hemineuromere in medial layers of microscopic stacks. **dashed line**; midline.

In the late embryo *sim* is maintained in the abdomen in a single stripe along the ventral median area of the embryo observed before (Fig. 3-6 D, H, L). Unfortunately, the thickness of the prosomal tissue and formation of cuticle at the end of embryogenesis impairs penetration of the probe, which made it impossible to analyse *sim* expression in anterior regions of late embryonic stages satisfactorily. In some embryos a very faint signal could be seen. After dorsal closure when the two germ band halves have migrated towards ventral again and the embryo has closed at the ventral midline expression of *sim* was seen in a stripe along the seam (Fig.3-6 L).

3.2.2 *Sim* is expressed in a region that does not give rise to neural cells

To address the question whether neural cells arise from the *sim* expressing region I combined *sim in situ* hybridisation with antibody stainings against Cadherin and Tubulin at a developmental stage where most of the NPGs have formed in the NE of *Achaearanea* (Fig. 3-9 A). The triple staining reveals that expression of *sim* does not coincide with the expression of either of the markers. Higher magnifications of single hemisegments clearly show the individual NPGs in the NE marked by anti-Cadherin and/ or anti-Tubulin, but none of these are also *sim* positive (Fig. 3-9 F-H). *Sim* therefore does not seem to be expressed in the actual NE, at least not in the region where the neural precursor cells are recruited, but immediately next to it.

These findings suggest that the region where *sim* is expressed in *Achaearanea* is neither part of the neural tissue or NE, nor was any expression of *sim* found in the transiently existing ventral midline epithelium itself.

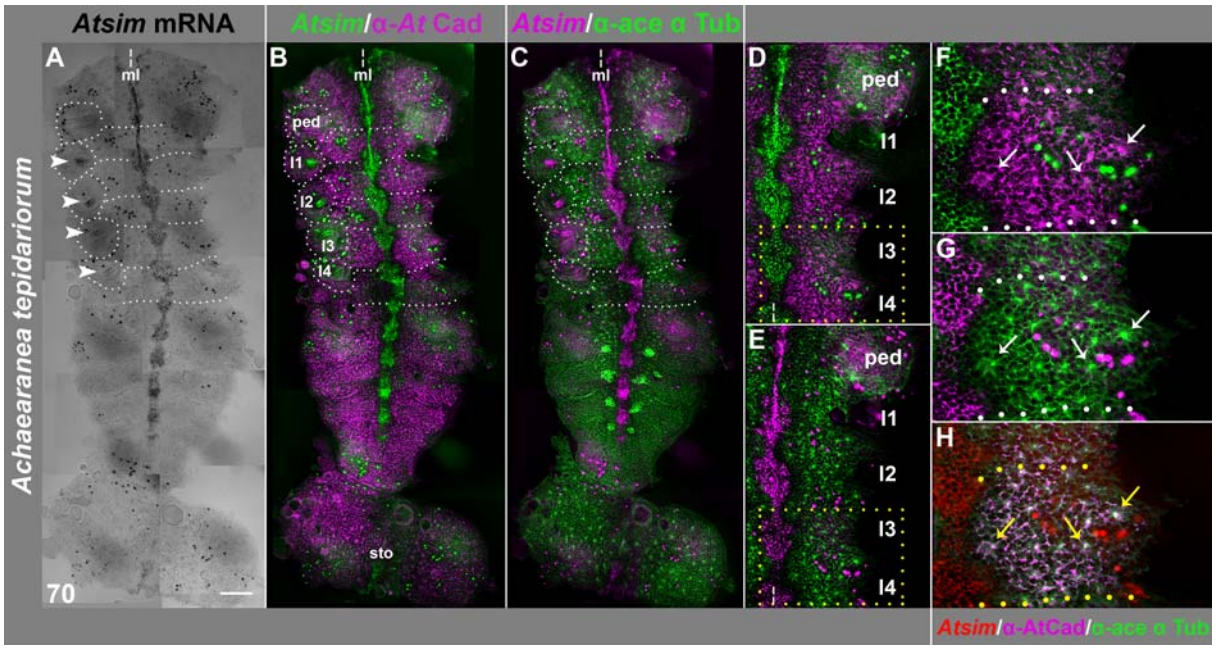

Figure 3-9: *Single minded* is not expressed in neural precursor cells.

Flat preparation of an embryo of *Achaearanea* before inversion at 70h of development, triple stained with Dig-labelled *At sim* RNA antisense probe and the antibodies *At* Cadherin and acetylated α Tubulin (**A**-**C**). Higher magnification of the leg segments of one embryonic half (**D**, **E**). Close-up of a single hemisegment (**F**-**H**). (**A**) *Sim in situ* hybridisation alone. *sim* is expressed along the ventral median area of the embryo. At the segment boundary (white dotted line) the germ band is slightly constricted, here expression of *sim* is broadest. Additional *sim* signal was detected in the bases of the four walking legs (arrowheads). During the preparation procedure the head got severed from the prosoma and is attached to the abdomen. (**B**) Double staining of the same *in situ* combined with Cadherin (magenta). The signal of the *in situ* against *sim* is converted to a complementary colour (green) for better depiction. (**C**) The same embryo, double staining shown for *sim* RNA (magenta) and Tubulin (green). (**F**) Magnification of one hemisegment shows double staining with the *sim* probe and against the Cadherin antibody. None of the invagination groups marked with Cadherin (arrows) show *sim* signal. (**G**) The same is true for the double staining of *sim in situ* and Tubulin antibody staining. The NPGs that show Tubulin expression (arrows) do not express *single minded*. (**H**) Merge of all three stainings combined. *sim* expression (red) is excluded from the groups of neural precursor cells in the PNGs (yellow arrows). **ch**; chelicera; **ml**; midline, **l1-l4**; leg 1-4, **ped**; pedipalp, **sto**; stomodeum, the scale bar is 100µm

3.2.3 *Sim* does not play a role in the process of inversion

In a next step I wanted to see which role *sim* plays in embryogenesis of *Achaearanea*. As shown previously it is expressed neither in the actual NE where the neural precursors are formed nor in the epithelium of the ventral embryonic region. Does down-regulation of *sim* transcript in the embryo have an effect on the process of inversion? To address this question I used RNAi mediated depletion of the *sim* gene product. In control experiments ds GFP was injected into the embryos. 302 *sim* RNAi embryos of *Achaearanea* were analysed under the aspect if the process of inversion was taking place correctly. About 23% of the embryos do not develop

properly as a result of the injection procedure, about the same number was misdeveloped in the control embryos injected with ds GFP RNA. The remainder of 74% (226) of *sim* RNAi treated embryos show a completely normal embryogenesis up to mid-inversion. Therefore it can be assumed that *sim* is at least not involved the correct progression of midline formation and inversion. Due to fixation problems immunohistochemic approaches did not work very well and unfortunately further experiments on *sim* function in RNAi embryos had to be abandoned.

3.4 *Netrin* function in axon pathfinding and midline guidance in spiders

3.4.1 Isolation of *netrin* in spiders

Netrin proteins are a family of laminin related secreted proteins that are evolutionary highly conserved. They comprise in all cases of an N-terminal signal peptide, two domains homologous to laminin containing three EGF-repeats and a C-terminal region, where the highest degree of variation is found (see appendix, Serafini et al., 1994). Conserved sequence sections of Netrin family members found in various species were used to amplify PCR fragments via cDNA cloning using degenerate oligonucleotide primers. I successfully cloned one *netrin* homologue in *Cupiennius* (Fig. 3-10, for the full alignment see appendix). In *Achaearanea netrin* was isolated using the same degenerate or specific primers, which were complementary to the sequence obtained for *Cs netrin*. The sequences of both spider species are almost perfectly identical (see appendix). To analyse the pattern of *netrin* expression in both spiders I have designed DIG-labelled mRNA antisense probes for both *Cupiennius* and *Achaearanea netrin* and performed *in situ* hybridisation experiments.

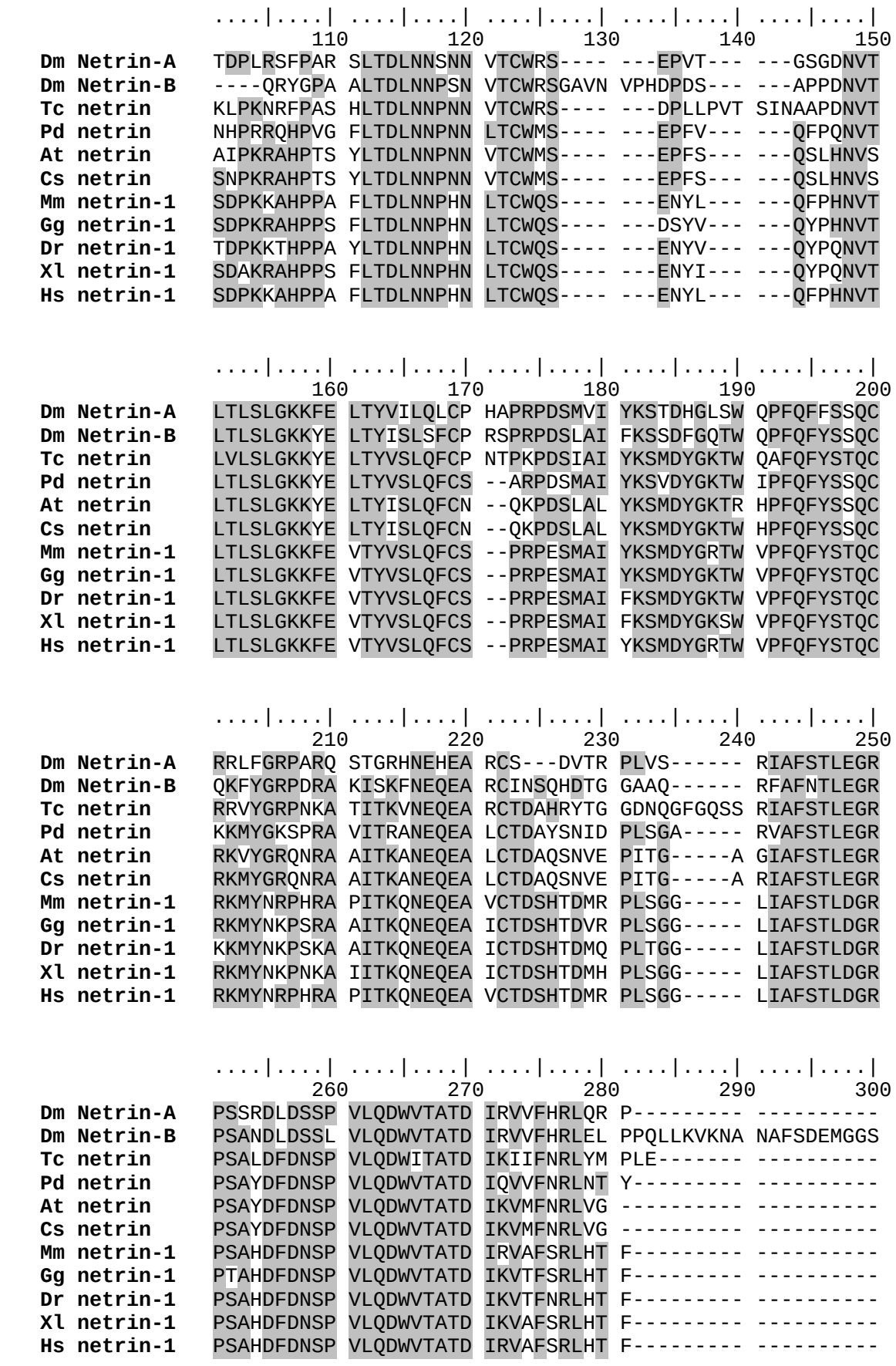

Figure 3-10: Amino acid alignment of the conserved region of *netrin* in various species.

Legend see next page (62).

Legend to Figure 3-10: The complete alignment can be found in the appendix. Amino acids that are identical in more than 6 species are highlighted in grey. ***At***; *Achaearanea tepidariorum*, ***Cs***; *Cupiennius salei*, ***Dm***; *Drosophila melanogaster*, ***Dr***; *Danio rerio*, ***Gg***, *Gallus gallus*, ***Hs***; *Homo sapiens*, ***Mm***; *Mus musculus*, ***Tc***; *Tribolium castaneum*, ***Pd***; *Platynereis dumerilii*, ***Xl***; *Xenopus laevis*.

3.4.2 *Netrin* expression shows two distinct domains in *Cupiennius*

In *Cupiennius* the transcript of *netrin* can be detected as early as 130h AEL. At that stage the first neural precursor cells are generated (Stollewerk et al., 2001), but the formation of commissures and scaffolding of the neuropile is not yet initiated. Expression of *netrin* was found in the entire developing nervous system, the cephalic lobes, the cheliceral, pedipalpal and the four leg bearing segments of the prosoma as well as in all segments of the opisthosoma (Fig. 3-11 A-D, Fig. 3-12 A). At first *netrin* expression can be seen in each segment in a ring-like pattern. These rings of *netrin* expressing cells encircle the individual invagination sites. In cross-sections through an individual hemineuromere this is even more obvious. The *netrin* positive sheath is formed like a pocket in which the neural precursor cells reside (Fig. 3-13 B). Within each hemisegment the *netrin* positive cells cover the entire width of the neuromere with the strongest expression in the medial to posterior area (Fig. 3-12 A, E, I). A second expression domain, which shows a weaker *netrin* signal, lies more anterior medially in the hemisegment, adjacent to the segmental furrow. Expression of *netrin* in these cells lacks the ring-like arrangement and expression in this area is distributed rather uniformly. The *netrin* positive cells of this second domain are located exactly where axonal projections will leave the NE to cross the midline (Fig. 3-12 A, E, I; Fig. 3-13 C). According to the anterior-posterior gradient of germ band development, the prosomal segments show the same degree of maturation, while the posterior segments lack behind. Segments of the opisthosoma are smaller and less differentiated the more posterior they are located and less invagination sites have formed. The *netrin* expression pattern is consistent with this finding as the sheath-like pattern observed in all segments is much less pronounced in opisthosomal segments (Fig. 3-12 A). Strong expression of *netrin* can also be found in the cephalic lobes. Here *netrin* is also expressed in a ring-like pattern.

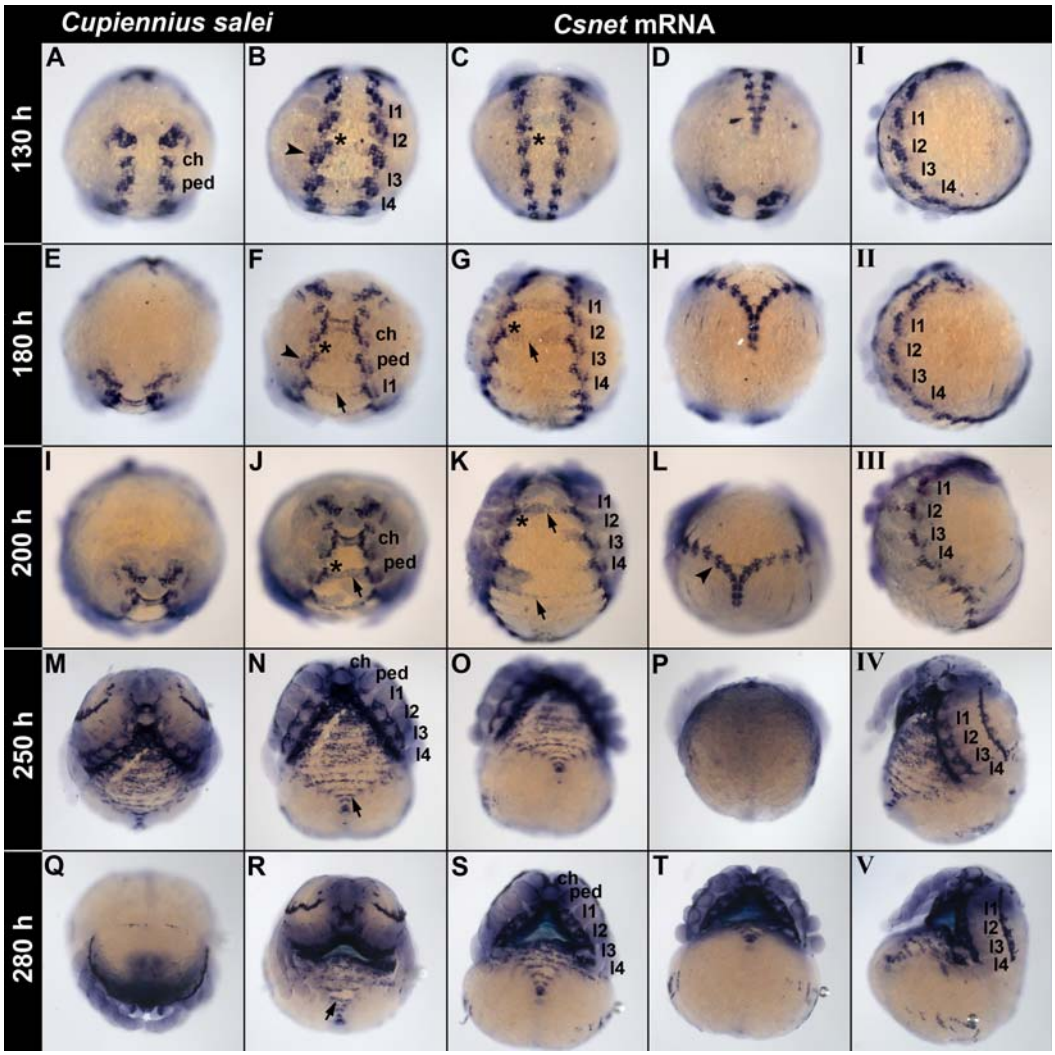

Figure 3-11: Expression of the *netrin* mRNA in whole mount embryos of *Cupiennius salei*.

Whole mount embryos of *Cupiennius salei* at different developmental stages hybridised with the DIG-labelled antisense RNA probe of *Cs netrin*. The different developmental stages give an overview over the dynamic changes of *netrin* expression during development of the embryo from early stages of nervous system development right to the end of embryogenesis. **A-V** show ventral views of the embryo from anterior to posterior. **I-V** show lateral views of the embryos. (**A**-**D**) Early in neurogenesis when the NPGs are formed *netrin* is expressed in the entire neuroectoderm of each segment (arrowhead). The signal is strongest in a band diagonally in the hemisegment. The second domain of *netrin* expression is marked by an asterisk. This domain is exactly positioned where the commissures will form to cross the ventral midline. (**E**-**H**) Just a little later, with the onset of inversion at around 180h, the first bands of *netrin* expressing cells can be identified in the prosoma spanning the midline (arrows). The expression of *netrin* in the sheath within segments seems already less strong. (**I**-**L**) During inversion the commissures have formed and *netrin* can be seen in them as well (arrows). In the opisthosoma the sheath like expression pattern is still evident (arrowhead). (**M**-**P**) At dorsal closure at 250h of development the prosoma is already covered with cuticle, thus giving strong background staining. However in opisthosomal segments the *netrin* positive commissural tracts that span the entire midline epithelium are clearly visible (arrow). (**Q**-**T**) Towards the end of embryogenesis when the midline becomes reduced again and the commissures shorten with it, *netrin* is still strong in these (arrow). Due to the constriction they appear to be even broader. **ch**; chelicera; **I1-I4**; walking leg 1-4, **ped**; pedipalp.

With the beginning of inversion cells of the ventral midline epithelium start to express *netrin* in transverse stripes that reach across the gap between the germ band halves. These bands of *netrin* positive cells reach from the point where axons will grow out to build up the commissural tracts of one embryonic half to the other (Fig. 3-11 F, G, J, K). Cross-sections through the area of commissural growth reveal that the cytoplasmic *netrin* expression belongs to cells of the single layered midline epithelium (Fig. 3-13 D).

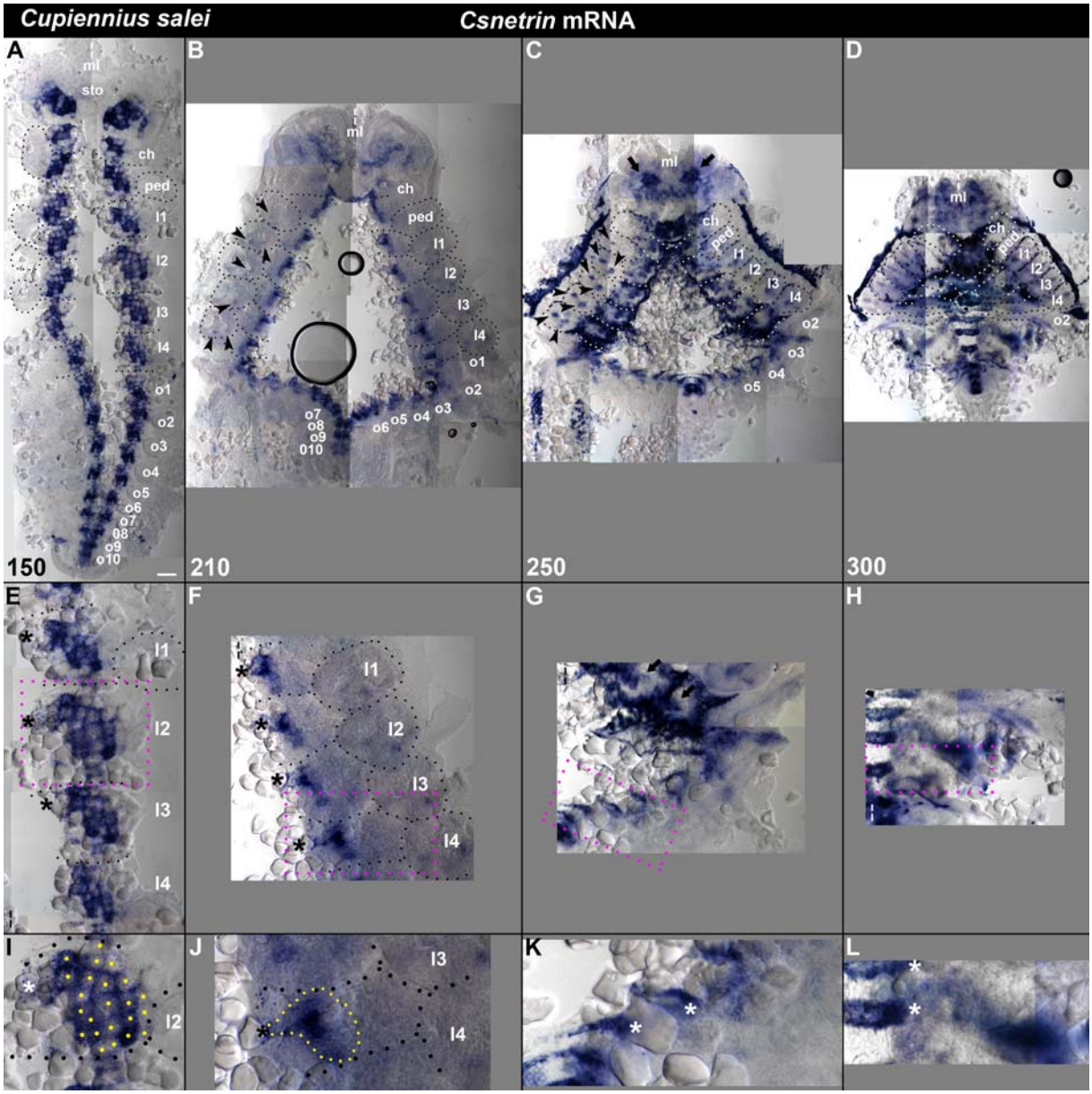

Figure 3-12: *Netrin in situ* hybridisations in *Cupiennius salei* embryos show two distinct domains of expression.

Legend see next page (65).

Legend to Figure 3-12: The panel shows *in situ* hybridisations with the DIG-labelled *netrin* RNA antisense probe generated in *Cupiennius*. **A**-**D** Flat preparations of complete *Cupiennius* embryos at different developmental stages give an overview of *netrin* expression over time. **E**, **F** close-up of the leg bearing segments of one half of the embryo. **G**-**H** close-up of opisthosomal segments of one half of the embryo. **I**-**L** Magnifications of individual hemineuromeres. (**A**) Expression of *netrin* comes up even before commissures are beginning to form and covers most of the NE and a broad domain in the head. *Netrin* positive cells are arranged into a ring-like pattern. (**B**) When inversion starts and both germ band halves move towards lateral and dorsal, expression of *netrin* becomes reduced to a domain at the anterior medial margin of the NE. In the developmentally younger segments of the opisthosoma, the ring pattern is still discernable. Expression in the head is lighter and has migrated towards more anterior positions. Additionally *netrin* positive cells were also seen in the bases of the walking legs in clusters of cells (arrowheads). (**C**, **G**) At dorsal closure, when the midline epithelium is maximally expanded *netrin* expression is difficult to observe due to formation of cuticle. Where recognisable *netrin* is found in small cell clusters anterior-medial in the NE (arrow in **G**). Expression of *netrin* in the head is now very strong in two symmetrically arranged cell clusters (arrows). The expression in the legs is clearly visible in two separate areas (arrowheads). (**D**) Shortly before ventral closure strong expression of *netrin* can be seen in the commissures, especially in the opisthosoma. (**I**) In the close-up the ring-like *netrin* pattern that surrounds the invagination sites of the hemisegment is notable. The *netrin* expressing cells encircle the NPGs like a sheath. The position of the *netrin* rings exactly correspond with the positions of the invagination groups within the segments, which are marked by yellow dots. Besides this broad *netrin* domain, there is a second albeit weaker domain located at or near the exit of the NE (asterisk). (**J**) The sheath like domain is no longer visible in the NE. Instead *netrin* expression has become reduced to a band of cells at medial positions within the NE (yellow dotted line), with the strongest expression anterior, close to the segment boundary and adjacent to the point (asterisk), where commissural tracts leave the nerve cord to cross the midline. (**K**) Since the prosoma is mostly covered in cuticle that gives strong background staining and hinders the probe from entering the tissue, specific *netrin* expression is difficult to identify. In the opisthosoma *netrin* can be found in the region where commissures grow out from the neuromere (asterisk) and in contralateral tracts themselves. (**L**) The commissures crossing the midline are *netrin* positive and very broad. Where they leave the NE, *netrin* positive cells are still to be seen (asterisk). **ch**; chelicera; **l1-l4**; leg 1-4, **o1-o10**; opisthosomal segment 1-10, **ped**; pedipalp, **sto**; stomodeum, the scale bar is 100µm.

After about 200h of embryonic development, almost midway through inversion, differentiated neurons have already left the invagination groups and start to build axonal projections (Döffinger and Stollewerk, 2010; Stollewerk et al., 2001). *Netrin* expression within the segments now is less strong than in the younger stages. Cells that are *netrin* positive become restricted to more or less one domain anterior medially. The strongest concentration of *netrin* transcript is found close to where the commissures exit the NE. The *netrin* expression posterior to it is less distinct. The ring-like pattern of *netrin* expression seen earlier, is no longer evident (Fig. 3-12 B, F, J). As formation of the axon scaffold takes place, commissural tracts cross the midline and were found to be also *netrin* positive, which is best seen in whole mounts (Fig. 3-11 F-H, J-M, O and P). Inversion progresses and the commissural tracts follow the morphological movement of the germ band halves. They expand dorso-ventrally during the separation and are spread across the entire midline epithelium. *Netrin* is expressed in the commissures throughout this process (Fig. 3-11 G, L, P, T, X). Sections through developmentally older segments reveal that indeed not only cells of the midline epithelium express the *netrin* transcript but also commissural

fibres (Fig. 3-13 E, F). All together I counted 6 prosomal and 8-9 opisthosomal segments starting with o2 (o2-o10), that show expression of *netrin*. The head expression of *netrin* has been split into several groups and is rather weak compared to the stage before inversion (Fig. 3-12 B). Additionally to the *netrin* expression related to the CNS in the bases of the pedipalps and the walking legs, a weak *netrin* signal was observed in one to two separate cell clusters (Fig. 3-12 B).

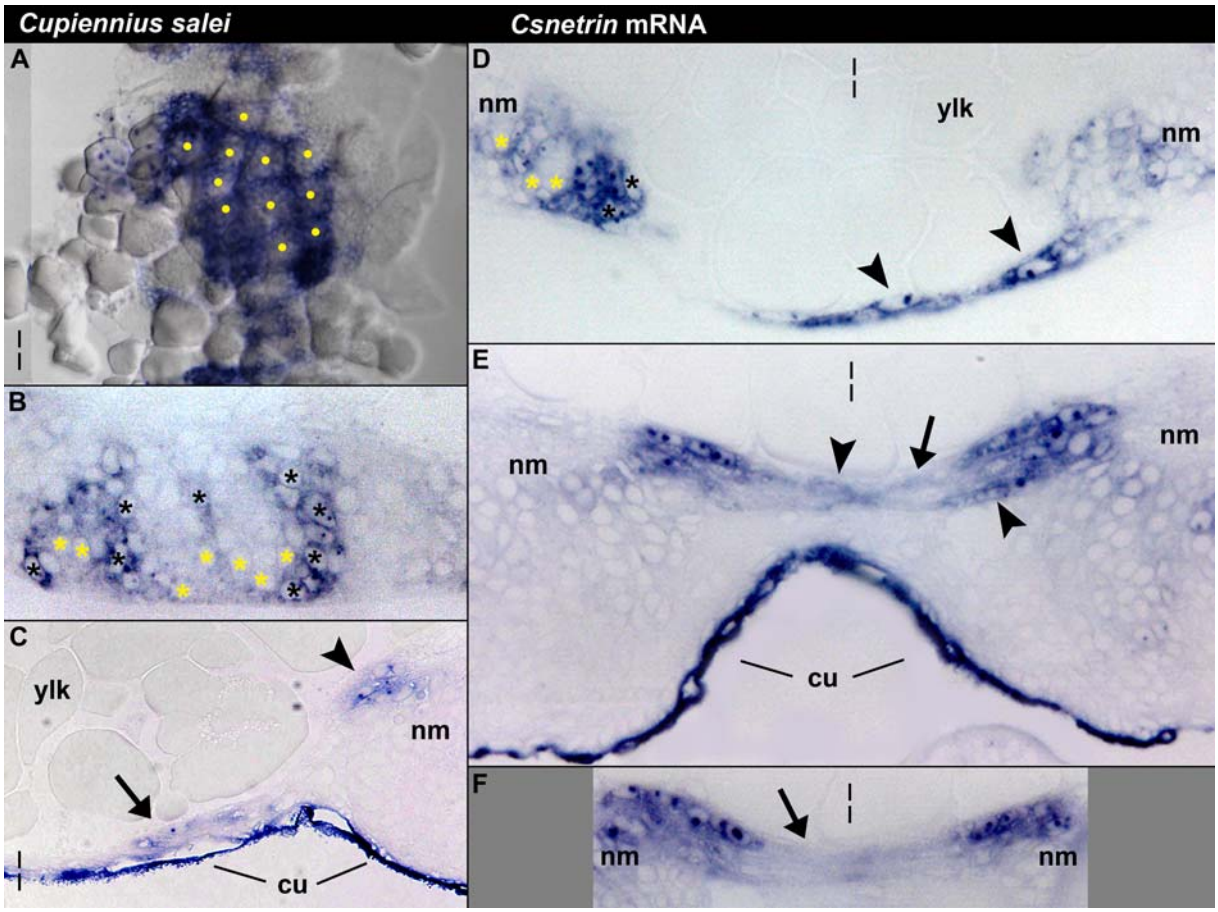

Figure 3-13: Sections of *netrin in situ* hybridisations in *Cupiennius salei* embryos show two distinct domains of expression.

In situ hybridisation on *Cupiennius* embryos with the DIG-labelled *netrin* RNA antisense probe. (**A**) Higher magnification of an individual leg hemineuromere. *Netrin* positive cells are arranged in a ring-like pattern surrounding the invaginations sites. The average position of the NPGs is indicated by yellow dots. (**B**) Cross-section through a hemineuromere. *Netrin* expression is detected in cells (black asterisk) that ensheath cells of the invagination groups (yellow asterisk). (**C**) Cross-section through the region where commissures leave the NE to cross the midline. *Netrin* is expressed in cells of the exit area (arrowhead) where commissural fibres (arrow) grow out from the neuropile. (**D**-**F**) Sections through progressively older segments of one embryo. (**D**) Initially cells of the ventral midline epithelium express *netrin* (arrowheads). In the NE sheath cells can be observed that are *netrin* positive (black asterisk) and surround neural precursor cells (yellow asterisk). (**E**) In a developmentally older segment besides the *netrin* positive cells of the midline epithelium and cells in the neuromere (arrowheads) also the commissural projections show *netrin* signal (arrow). (**F**) Finally, only the fascicles crossing the midline express *netrin* (arrow). Cytoplasmic expression is visible in neuroectodermal cells. **cu**; cuticle, **nm**; neuromere, **ylk**; yolk.

At dorsal closure after around 250h of development *netrin* expression in the prosoma is difficult to distinguish from the strong background staining caused by the cuticle, which is beginning to form at around 230h. In the abdomen however (the formation of cuticle shows a gradient from anterior to posterior) *netrin* expression can be seen in the same region as before, the anterior-medial most corner of the hemisegment. The size of the domain is adjusted to the opisthosoma. The commissural tracts are *netrin* positive as was the case before (Fig. 3-12 C; Fig. 3-13 F). In the head region *netrin* is restricted to one prominently stained area and some scattered cell clusters, some of which still show the ring-like organisation (Fig. 3-12 C). Apart from the nervous system, *netrin* was found to be expressed in the bases of the pedipalps in one cell cluster and in two clusters in the walking legs (Fig. 3-12 C).

During late embryogenesis most of the prosoma is covered in cuticle making it impossible to identify a specific *netrin* signal. In the opisthosoma it can be observed that *netrin* expression is still strong in the commissures. When the ventral midline becomes restricted again they appear as broad bands (Fig. 3-12 D).

Taken together, from early stages in nervous system development in *Cupiennius* one can distinguish two different expression domains of *netrin* in the ventral NE. The first one is a broad centrally located expression domain, where *netrin* positive cells ensheath individual invagination groups and secondly a medial domain, which correlates with the exit point of commissural axons.

3.4.3 *Netrin* expression in *Achaearanea*

Compared to *Cupiennius* in *Achaearanea* I observed that the pattern of *netrin* expression is only partially comparable. The ring-like pattern of *netrin* expression was not detectable in *Achaearanea*. However the expression of *netrin* in the neuropile region of commissural outgrowth, in cells of the ventral midline epithelium and the commissures themselves appears to be identical in both species.

In *Achaearanea* expression of *netrin* can first be observed at around 75h in clusters of cells, which lie anterior medially within the hemisegment (Fig. 3-14 A, E, I, the

weak staining in some of the leg segments in A is due to poor penetration of the probe into the tissue since the legs cover the NE very tightly).

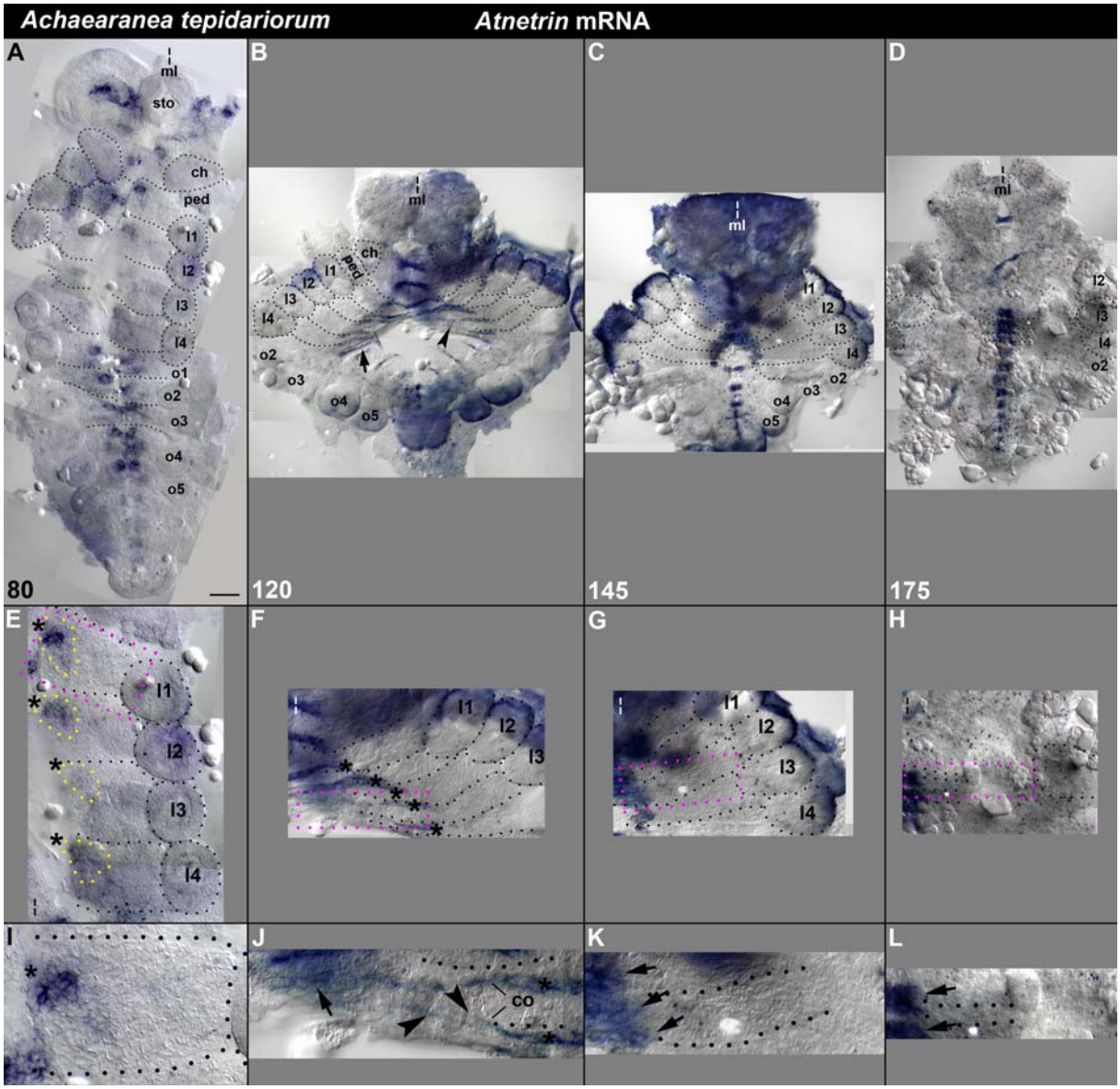

Figure 3-14: *Netrin* expression in embryos of *Achaearanea tepidariorum* mirrors only one of the domains observed in *Cupiennius*.

Flat preparations of *Achaearanea* embryos hybridised with an *At netrin* RNA antisense probe that was DIG-labelled. **A**-**D** Complete flat prepped embryos of *Achaearanea* give an overview of the temporal changes in *netrin* expression. **E**-**H** show magnifications of the four leg hemineuromeres. **I**-**L** depict one single hemisegment in more detail. (**A**) During the hybridisation procedure the legs, that had not been removed prior to the *in situ*, had covered the prosoma resulting in the weak staining observed. Still *netrin* expression can be seen, especially in the opisthosoma, in a cell cluster anterior medial in the segment close to the segment boundary. Additionally *netrin* expression was also found in the head. (**B**) At mid-inversion the *netrin* positive cell clusters have stretched into bands of cells (arrow). Also the commissures that cross the midline show strong expression of *netrin* (arrowhead). (**C**) When the embryo is closed dorsally and the midline epithelium is narrowed down to a small patch, *netrin* expression of both embryonic halves fuse. (**D**) This is even more obvious after ventral closure and complete reduction of the midline. At the end of embryogenesis *netrin* is expressed in one broad stripe-like domain in each segment.
Legend continues next page (69).

Legend to Figure 3-14: (**I**) In the magnification of a single leg hemisegment it can be seen that the *netrin* expressing cells are positioned close to the region of the NE, where commissures will start to grow out to cross the midline (asterisk). The bigger part of the neuromere appears to be free of *netrin* expression. (**J**) The *netrin* expressing cell cluster has changed into a stripe like pattern. In the close-up it is obvious that the strong *netrin* expression across the midline stems from a stripe of midline epithelial cells (arrows). Nevertheless I also observed commissural fibres that grow out from the NE and extend across the midline and are *netrin* positive (arrowheads). (**K**) Towards the end of inversion not only the *netrin* domains of symmetrical hemisegments have fused where the midline has been reduced, also in anterior-posterior direction clusters of neighbouring hemisegments seem to have merged. (**L**) Finally, at ventral closure and extinction of the ventral midline one strongly expressing cluster of *netrin* positive cells can be seen in each segment. **ch**; chelicera; **l1-l4**; leg 1-4, **o1-o10**; opisthosomal segment 1-10, **ped**; pedipalp, **sto**; stomodeum, the scale bar is 100µm.

During inversion the *netrin* expression domain is modified from the cell cluster pattern to a stripe-like pattern, since now also cells of the ventral midline tissue express *netrin*. These bands of *netrin* positive cells span the entire breadth between the germ band halves (Fig. 3-14 B, F, J). The more they move towards lateral and dorsal positions the longer the *netrin* bands become. Furthermore, I also observed *netrin* expression in fibrous structures that project contralaterally, presumably the commissures (Fig. 3-14 J). Double stainings, which combine the *netrin in situ* hybridisation with an antibody staining against Cadherin reveal that the *netrin* positive cells are positioned immediately adjacent to the neuropile, where the commissures form and leave the NE (Fig. 3-15 B-D). Axons, which grow out from the neuropile to project contralateral to establish the commissures, are directed towards them. Across the midline the commissures then follow the band of *netrin* expression in the midline epithelium (Fig. 3-15 D).

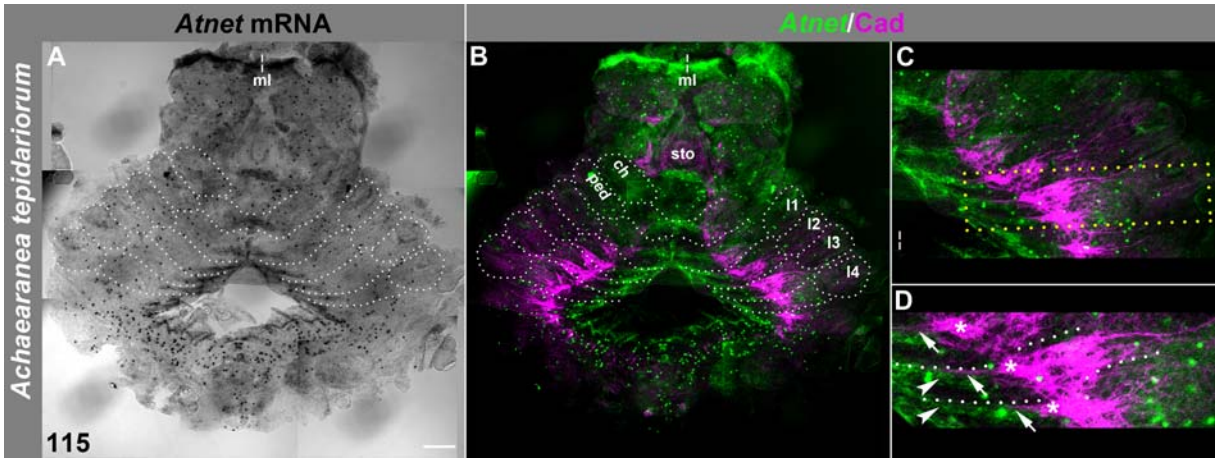

Figure 3-15: Commissural projections grow towards the *netrin* positive cells and follow them across the midline.

Legend see next page (70).

Legend to Figure 3-15: Flat preparation of an embryo of *Achaearanea* at mid-inversion after 115h AEL double stained with Dig-labelled *At netrin* RNA antisense probe and the antibody against *At* Cadherin (**A**, **B**). Magnification of the leg segments of one half of the embryo (**C**). Close-up of a single hemisegment (**F**-**H**). (**A**) Composition of basal layers of the NE of an *in situ* hybridisation with the *At netrin* probe alone. *Netrin* is expressed in segmental stripes within the segments. These stripes are somewhat diagonally orientated and reach from medial positions of the hemisegment to the anterior-medial area, where the commissures grow out. (**B**) Double staining of *netrin* probe (shown in green) and the Cadherin antibody (magenta). For Cadherin maximum projections of basal confocal sections are shown. It is clearly visible how the *netrin* positive cells are positioned right next to the neuropile from where axonal projections grow out to cross the midline. (**C**) Magnifications of the leg segments of one half of the embryo. (**D**) In the close-up of one individual hemisegment arrows indicate the commissures that grow out from the NE along the bands of *netrin* expressing cells, which lie adjacent to the neuropile (asterisk) and then provide a *netrin* source across the midline. **ch**; chelicera; **l1-l4**; leg 1-4, **ped**; pedipalp, **sto**; stomodeum, the scale bar is 100µm.

In late embryogenesis the midline becomes minimised again after dorsal closure as the two germ band halves of the embryo move towards each other on the ventral side. Where the embryo has closed already (the anterior and posterior most segments) *netrin* expression is detectable in a single cluster per segment, the expression domains of both hemisegments appear to have fused. However, even in segments where the midline is not yet completely reduced, the band-like expression is no longer evident. *Netrin* positive cells are now found in clusters with cell groups of neighbouring hemisegments directly next to each other (Fig. 3-14 C, G, K). Shortly after that at around 175h of development and the end of embryogenesis, in all segments *netrin* expression is restricted to one cluster per segment, the broadest of which is seen in the prosoma (Fig. 3-14 D, H, L). I also performed double staining with Cadherin (Fig. 3-16). In the opisthosoma it can be seen that the *netrin* clusters are positioned immediately adjacent to the neuropile, in between the symmetrical longitudinal tracts. The commissures are located at the same position (Fig. 3-16 C, D).

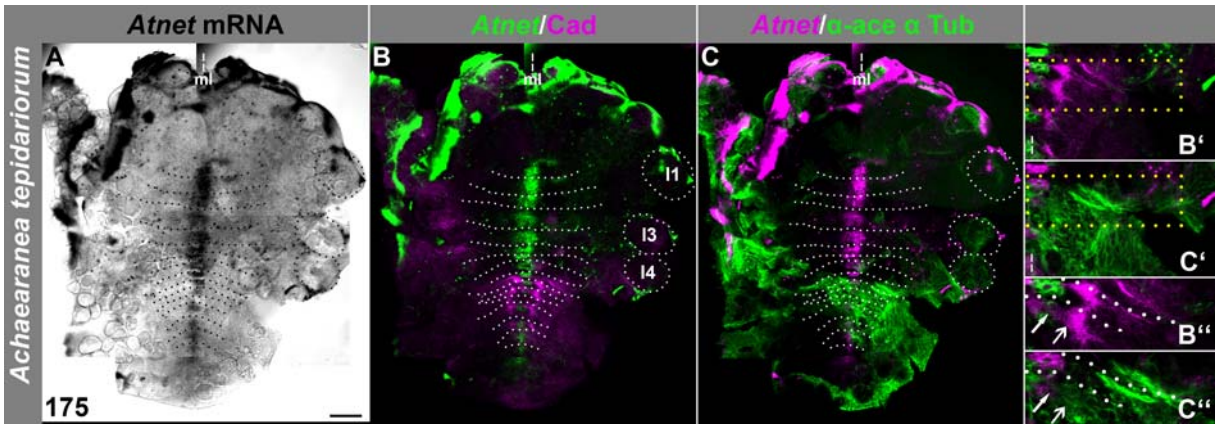

Figure 3-16: The *netrin* clusters fuse at ventral closure, commissures still follow the expression of *netrin* and shorten again.

Flat preparation of an *Achaearanea* embryo at the end of embryogenesis after 175h of development double stained with Dig-labelled *At netrin* RNA antisense probe and the antibody against *At* Cadherin (**A**, **B**). Magnification of opisthosomal segments of one half of the embryo, where the antibody staining worked (**C**). Close-up of a single hemisegment (**F**-**H**). (**A**) Flat preparation of an *Achaearanea* embryo with the signal of the *in situ* hybridisation alone. The two symmetrical *netrin* domains of the hemisegments of both embryonic halves have fused where the germ band halves have met at the reduction of the ventral midline epithelium. A single band of *netrin* expression can be seen in each segment. (**B**) The panel shows double staining of the *netrin in situ* (green) combined with a following Cadherin antibody staining (magenta). (**C**) shows the magnification of several segments of the opisthosoma. (**D**) Magnification of one individual segment. The *netrin* expressing cells of both germ band halves have fused ventrally to form a single cluster (arrow). The commissural fascicle (arrowhead) can be seen lying in the same area. **ch**; chelicera; **l1-l4**; leg 1-4, **ped**; pedipalp, **sto**; stomodeum, the scale bar is 100µm.

Taken together, although *Cupiennius* and *Achaearanea netrin* are almost identical in sequence, I observed different modes of *netrin* expression with respect to the ring-like expression in the two spider species. However, expression of *netrin* in the region of the neuropile, where commissures exit to cross the midline seems to be concordant in *Cupiennius* and *Achaearanea*. Additionally I could show that during inversion *netrin* is expressed continuously in transverse stripes in the ventral midline epithelium. Commissural fibres follow this *netrin* expression and are *netrin* positive themselves.

3.5 Knockdown of *netrin* function impairs axon pathfinding

As mentioned above *netrin* involvement in axon pathfinding and guidance at the midline is highly conserved across species. To investigate this and other potential functions of *netrin* in spiders, knockdown experiments using the embryonic RNA interference technique were applied and different aspects of neurogenesis and axon

pathfinding analysed. To evaluate whether RNA interference-mediated depletion of *netrin* transcript in the embryo was successful, I performed *in situ* hybridisations against *netrin*, using probes that were also applied for the description of normal *netrin* expression (paragraph 3.4.2). In control experiments ds GFP RNA was injected. Cadherin was used as a marker to analyse axon pathfinding and to see whether the establishment of the embryonic nervous system is impaired.

3.5.1 The levels of *netrin* expression is transiently reduced by injection of double stranded *netrin* RNA

The reduction of *netrin* transcript levels within the tissue is not consistent in all embryos injected. This is due to the variation of the amount of injected ds RNA and its effect on individual embryos treated. The development of eggs in one cocoon is not completely synchronised and embryonic stages can vary from a couple of hours up to one day. In embryos from a single cocoon that underwent the RNAi procedure at the same time, the developmental differences can be enhanced up to almost two days. Morphological examination of outer criteria such as overall embryonic development, germ band elongation and inversion showed that in both, control and *netrin* RNAi treated embryos, about 25% of embryos did not develop properly, if at all. These were discarded. Since the same amount of embryos showed these developmental defects, it can be assumed that this is due to the injection procedure as such and no side effect of the injected double stranded RNA. The injected embryos were left to develop up to a time point where the majority of them were of a developmental age of about 220h. They were fixed and underwent further analysis.

In situ hybridisations against *netrin* were performed on control and *netrin* RNAi treated embryos of the different stages at hand. In control embryos of the younger stages (n=4) the normal pattern of *netrin* expression can be observed as described above (Fig. 3-17 A, A'). In morphologically older embryos of the control (n=4) the *netrin* expression pattern of the ring-like domain is reduced within segments as is the case in untreated embryos. Expression of *netrin* in the region of commissural outgrowth looks entirely normal, besides that it is slightly weaker in the prosoma compared to the opisthosoma, which can be explained with the covering of the NE by

the legs that have hindered the hybridisation of the probe (Fig. 3-17 C, C'). In all developmentally younger stages of *netrin* RNAi embryos (n=4) *netrin* is clearly down-regulated as I did not detect any *netrin* signal in the NE right up to beginning of inversion. This is the case for both *netrin* domains. Neither the ring-like pattern of *netrin* expression can be seen, nor is the smaller anterior-medial domain detectable (Fig. 3-17 B, B'). However, in developmentally older embryos treated with RNAi (n=7), slight expression of *netrin* comes up during inversion. The position and distribution of this *netrin* positive domain, albeit weaker, looks very similar to that observed in control embryos of a comparable morphological stage. Also commissural projections growing out from the neuromere and the midline epithelial cells express *netrin*, though weaker than in the control (Fig. 3-17 D, D').

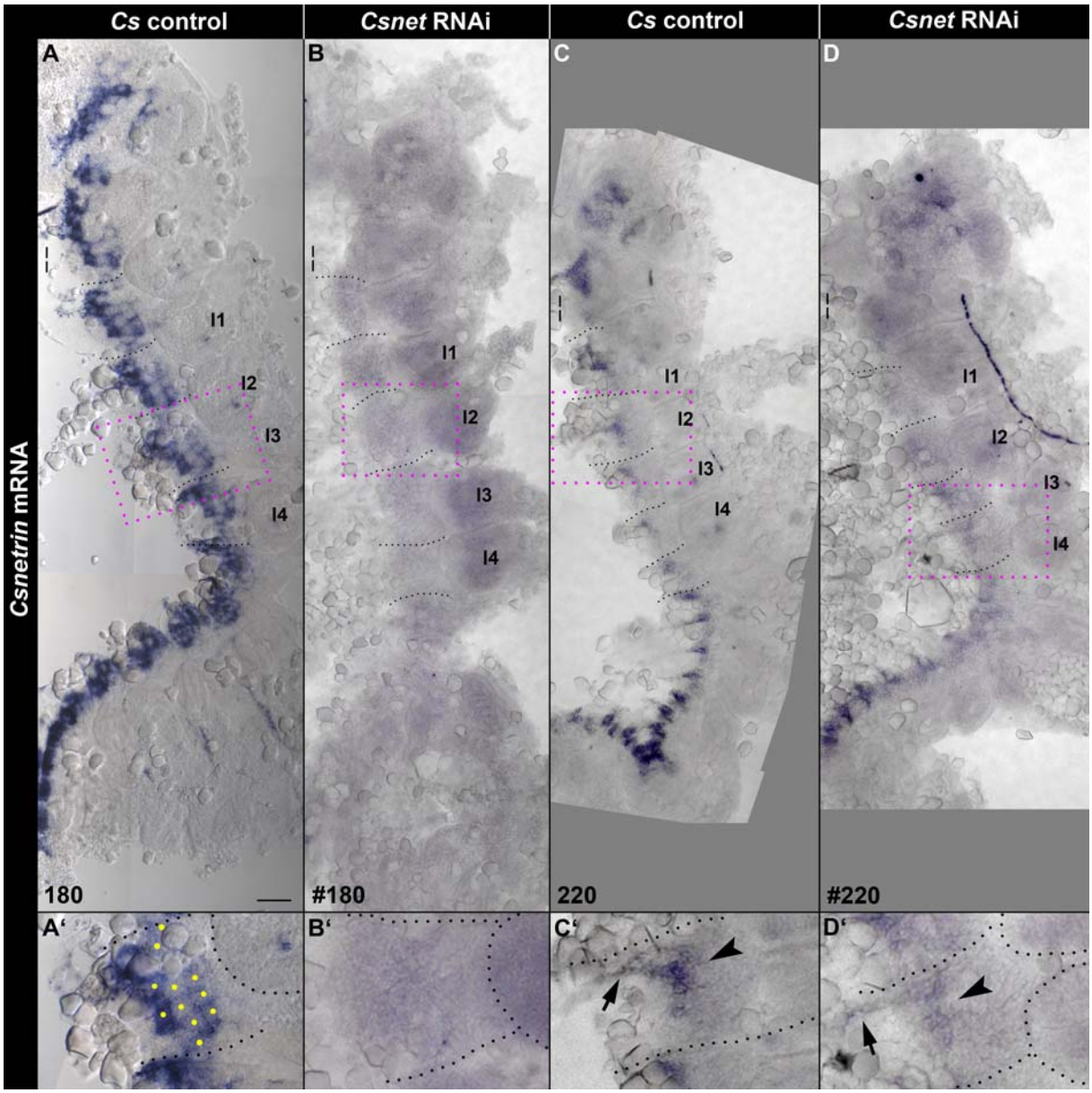

Figure 3-17: Verification of *netrin* down-regulation in *netrin* RNAi depleted embryos of *Cupiennius salei*.

The panel shows flat preparations of one germ band half of control embryos of *Cupiennius* injected with dsGFP (**A**, **A'**, **C**, **C'**) and *netrin* RNAi embryos (**B**, **B'**, **D**, **D'**). *Netrin in situ* hybridisations were performed to analyse the degree of down-regulation of endogenous *netrin* transcript by injection of double stranded *netrin* RNA into the egg. (**A**) In the control embryo at around 180h of development *netrin* is expressed normally in the NE. The typical ring-like pattern is visible. (**A'**) The magnification of one hemisegment shows that the arrangement of *netrin* positive cells is completely normal and *netrin* positive cells surround the invaginations sites (average positions marked by yellow dots). (**B**) Embryos treated with *netrin* RNAi at a comparable developmental stage show complete reduction of *netrin* transcript in the NE. (**B'**) Also in the close-up, no specific *netrin* expression can be detected. (**C**) After around 220h of development in the control embryo the ring-like expression domain is reduced as is the case in untreated embryos. Although the expression of *netrin* is rather weak in the leg segments (the legs cover the NE during hybridisation and prevent access of the probe to the tissue), the band of *netrin* expressing cells in the anterior-medial area of the hemisegment and in cells of the midline epithelium (arrow), is completely normal. (**D**, **D'**) Interestingly, *netrin in situ* hybridisation in RNAi treated embryos shows partially restored expression of *netrin* in exactly this domain. The signal is weaker than in control embryos but still detectable. **I1-I4**; leg 1-4, **dashed line**; midline, the scale bar is 100µm.

Taken together, in early stages of nervous system development expression of *netrin* seems to be sufficiently down-regulated in embryos injected with double stranded *netrin* RNA. However, during mid-embryonic stages, *net* expression in RNAi treated embryos is being restored to almost normal levels.

3.5.2 Down-regulation of *netrin* leads to defects in the formation of the embryonic axon scaffold

As described above in *Drosophila melanogaster* (Harris et al., 1996; Mitchell et al., 1996) as well as in vertebrates (Kennedy et al., 1994; Serafini et al., 1996; Serafini et al., 1994) *netrins* are expressed by cells at the CNS midline and are required for the formation of commissural pathways to connect the two symmetric sides of a nervous system. Disruption of both Net A and B in *Drosophila* lead to defects in commissure formation. Knockdown of *netrin* in vertebrates leads to defects in nervous system development as well.

To analyse in detail if and to what extend *netrin* is involved in the guidance of axons towards the midline and the formation of commissural tracts of the embryonic axon scaffold in spiders the Cadherin antibody was used. Is the down-regulation of *netrin* transcript, if only temporary, sufficient to impair normal axonogenesis? The focus here was on embryos at 220h of embryonic development, when in untreated embryos the neuropile is formed and the fascicles of the axonal scaffold are clearly established.

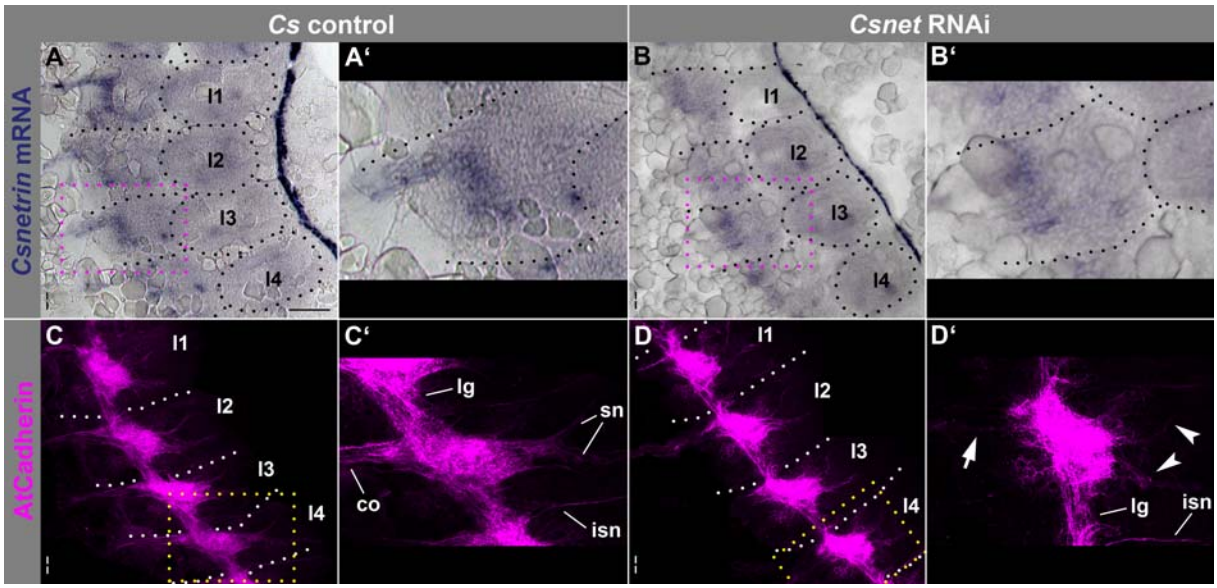

Figure 3-18: The correct formation of the embryonic axon scaffold is affected in embryos treated with *netrin* RNAi.

The panel shows the leg bearing segments of flat preparations of control embryos of *Cupiennius* injected with ds GFP at around 220h of embryogenesis (**A**, **A'**, **C**, **C'**) and *netrin* RNAi treated embryos of a stage accordingly (**B**, **B'**, **D**, **D'**) hybridised with Dig-labelled *netrin* RNA antisense probe (**A**, **A'**, **B**, **B'**) or stained against *At* Cadherin (**C**, **C'**, **D**, **D'**). (**A**, **A'**) In the control embryo at 220h *netrin* is expressed anterior-medial in the hemisegment. Also cells of the midline epithelium are *netrin* positive (arrow) to guide outgrowing commissures across the midline. (**B**, **B'**) Compared to the control *netrin* expression in *netrin* RNAi embryos is somewhat weaker but recognisable nevertheless. (**C**, **C'**) The embryonic axon scaffold develops normally in embryos injected with ds GFP. (**D**, **D'**) Scaffolding of the neuropile is affected in embryos injected with ds *netrin*. The establishment of the longitudinal axonal tracts and commissural connectives is impaired, although the individual neuromeres themselves are formed more or less normally. The arrow points to the commissure where some projections have formed although they are hard to see. Arrowheads indicate the position of the segmental nerve, which is difficult to identify. The longitudinal tracts appear to be less organised. Differences in the degree of malformation can be observed within one embryo as the leg hemisegments illustrate. **co**; commissure, **dashed line**; midline; **isn**; intersegmental nerve, **I1-I4**; leg 1-4, **lg**; longitudinal, **sn**; segmental nerve, the scale bar is 100µm.

Antibody staining against Cadherin in control embryos after 220h of development (hemisegments, n=12) confirm that the neuropile is established as described above (Fig. 3-18 C, C', compare with Fig. 3-1 C). RNAi treated embryos of an appropriate stage show that in 80% of the hemisegments analysed (n=41) the establishment of commissural tracts growing out from the neuromere is severely affected. Where recognisable, only a few projections are seen to cross the midline. In most cases though, commissural projections coming from the segmental neuropile are not formed at all. The longitudinal tracts appear to be thinner and less organised than observed in control embryos. The formation of the segmental nerve is also impaired when *netrin* is not present during early neurogenesis. Although axonal projections can be seen that grow lateral, their arrangement is more disorderly than in control embryos (Fig. 3-18 D, D'). This is an interesting finding, since *netrin* is normally not

expressed in the corresponding areas. However, the formation of the neuromeres itself does not seem to be impaired by the down-regulation of *netrin*. Their proportion and size in each hemisegment look rather normal (Fig. 3-18 D).

Consistent with its conservation across species, also in spiders *netrin* seems to function in axonal guidance. However, the phenotype is rather mild, which could be explained by the partial restoration of *netrin* expression during neurogenesis.

3.6 Knockdown of *netrin* function affects the differentiation state of neural precursor cells

In addition to the primary NPGs (pNPGs) secondary invagination groups (sNPGs) are formed in the VNC. This partially overlaps with the invagination of the pNPGs, which delaminate from the apical surface and differentiate between 200 to 230h. There is no clear temporal and spatial delimitation between the two. Already after 220h of development most of the sNPGs are visible while some of the primary groups still persist apically. Secondary invagination groups show specific features that clearly distinguish them from the pNPGs in several aspects (Stollewerk, 2004). For my studies the most interesting fact is that cells recruited to form sNPGs all detach from the apical neuroectodermal layers at about the same time. They then persist as large epithelial vesicles more medially within the segment. Individual cells of one site stay attached to each other and maintain their epithelial character, which might delay differentiation up to larval and possibly adult stages. This is in contrast to the pNPGs, which are formed sequentially. Differentiating cells steadily leave the cohesiveness of the compound and thus loose contact to the apical surface. Moreover, it was shown that sNPGs are surrounded by sheath cells that separate the individual invagination sites from one another. These sheath cells show the typical characteristics of glial cells, like cell shapes and processes (Stollewerk, 2004).

To see if there is a correlation between the formation of secondary invagination sites, the glia like nature of the sheath cells and expression of *netrin* in these, I first examined Phalloidin stainings at a stage when most if not all sNPGs have formed, to identify any changes in the formation and characteristics of secondary invaginations

sites. Secondly, to investigate aspects of neuronal differentiation, I looked at the differentiation marker *islet* (*isl*) and performed *in situ* hybridisations in *netrin* RNAi treated embryos at an equivalent developmental stage as the Phalloidin stainings.

3.6.1 Phalloidin stainings in *netrin* RNAi treated embryos of *Cupiennius* reveal that the morphology of the secondary invagination sites is altered

At 220h of embryonic development all of the secondary invagination sites are visible in individual hemineuromeres (Stollewerk, 2004). Phalloidin staining in control embryos at that stage confirm that they are arranged in the normal star-like pattern in all leg hemisegments analysed (n=20), with cell processes running in parallel so that they appear as long thin lines in confocal sections (Fig. 3-19 A, A'). Compared to this, in *netrin* RNAi treated embryos of *Cupiennius* at around 220h one can observe that the organisation and arrangement of sNPGs is affected. On the one hand the number and positions of groups formed seems to be unchanged, although they appear to be somewhat smaller. However, the star-like morphology within the neuromere is disturbed and the long cell processes characteristical for the secondary groups are not formed properly. The extensions are markedly shorter if visible at all (n=65; Fig. 3-19 B, B').

3.6.2 Down-regulation of *netrin* in *Cupiennius* leads to an increase of cells that express the differentiation marker *islet*

Reduction of the level of *netrin* transcript early in the spider embryo using the embryonic RNAi technique could show that the formation of the neuropile was affected. Although the neuromeres showed no apparent phenotype, the scaffolding between individual hemisegments via commissures and longitudinals and the innervation of the periphery was severely impaired. To analyse whether depletion of *netrin* function also has an effect on neuronal differentiation and cell fate, I looked at the differentiation marker *islet*. In *Drosophila islet* is expressed in motoneurons, which innervate the ventral muscles and in interneurons that were identified as

dopaminergic and serotonergic neurons (Thor and Thomas, 1997). Several neuroblasts were identified to generate *islet* positive cells (Landgraf et al., 1997; Lundell and Hirsh, 1998). Also in vertebrates, where these LIM domain molecules were first discovered, *islet* defines different classes of motor neurons (Appel et al., 1995; Pfaff et al., 1996; Tsuchida et al., 1994) with *Isl-1* serving as a marker. In *Cupiennius* seven NPGs were identified that generate *islet* positive cells (Döffinger and Stollewerk, 2010).

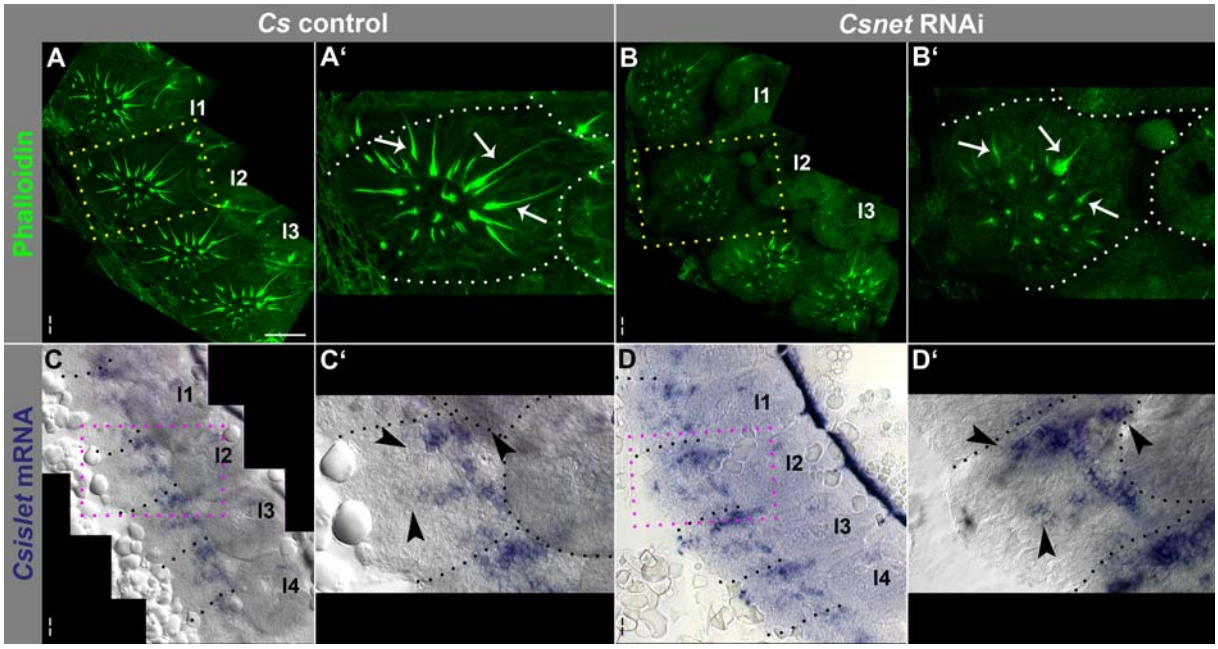

Figure 3-19: Down-regulation of *netrin* in *Cupiennius salei* leads to disturbance of the formation of the secondary invagination sites and defects in the expression of *islet*.

The panel shows the leg bearing segments of flat preparations of control embryos of *Cupiennius* injected with ds GFP (**A**, **A'**, **C**, **C'**) and *netrin* RNAi embryos (**B**, **B'**, **D**, **D'**) stained with Phalloidin (**A**, **A'**, **B**, **B'**) or hybridised with a *Cs islet* RNA antisense probe (**C**, **C'**, **D**, **D'**). (**A, A'**) In the control embryo at 220h the pattern and arrangement of the secondary invagination sites looks absolutely normal. (**A'**) The magnification of a single hemisegment reveals that the long protrusions (arrows) that are typical for the vesicles of the secondary precursor groups are formed as in WT. (**B**, **B'**) Compared to the control, embryos treated with ds *netrin* RNA show severe defects in the organisation of the secondary precursor groups. (B') The impression is that in fact the overall number of sites is similar to the control, but the star-like arrangement is disturbed. The long cellular extensions (arrows) seen normally are shortened dramatically or missing altogether. (**C**) The *islet in situ* in control embryos at 220h of development shows expression of the neural differentiation marker in the lateral part of the NE. (**C'**) In the close-up the *islet* expressing cells can be subdivided into several clusters. (**D**) In RNAi treated embryos, the number of *islet* positive cells in the NE has increased noticeably. (**D'**) Additional *islet* positive clusters (arrowheads) were found in each hemisegment. One lies more medially in the hemisegment, a second anterior close to the segment boundary and a third was seen more laterally. The remaining clusters that are also present in the control embryos seem to contain more *islet* expressing cells. **I1-I4**; leg 1-4, **dashed line**; midline, the scale bar is 100µm.

The expression pattern of *islet* is highly dynamic during embryogenesis of *Cupiennius salei*. In control embryos at 220h of development in each hemineuromere *islet* positive cells are distributed into several cell clusters, which are located in the anterior lateral part of the NE (Fig. 3-19 C, C'). All together an average of 44.12 cells can be counted per hemineuromere (n=12). In embryos where *netrin* was down-regulated by RNAi treatment in the hemisegments (n=52) I found an increase of *islet* positive cells (60.94) by about 27.6% (16.82 cells). These additional cells are found in positions where usually no *islet* expression is present. Also the cell clusters that would normally show *islet* signal seem to contain more cells (Fig. 3-19 D, D'). These findings suggest that in the absence of *netrin* in the NE and in particular in the glial sheath surrounding the invagination sites, the epithelial vesicles start to disintegrate earlier than normal. Cells of the precursor groups would then leave the compound to differentiate and adopt a specific cell fate at the expense of normally later differentiating neural cells. Furthermore, going through microscopic stacks, I could observe that the distribution of the cells within the neuromere in apical-basal direction is altered compared to control embryos. Due to the greater number of cells that express *islet* or are subject to an earlier differentiation, cells would compete with each other to position themselves. Instead of the cell columns in apical-basal direction observed in untreated and control embryos, they are spread out in more or less just a few layers of the NE (not shown).

Taken together these results suggest an altogether different role for *netrin* in spider nervous system development beside the conserved function in axon path finding.

4 Discussion

4.1 Several aspects of the development of the embryonic axon scaffold are highly conserved in arthropods

The typical embryonic nervous system in arthropods comprises of longitudinal axon tracts that interconnect the neuromeres of neighbouring hemisegments and commissures that run perpendicular to the longitudinal tracts (Paulus and Weygold, 1996; Bullock and Horridge, 1965). These connect the bilateral symmetrical sides of the VNC to relay information from one side to the other, resulting in a rope-ladder like structure. Additionally, peripheral nervous projections convey incoming and outgoing information. The mechanisms involved in establishing an axon scaffold are well understood in insects, especially in *Drosophila* and in the grasshopper (*Schistocerca gregaria*) and early axonogenesis was shown to be remarkably conserved in both. Some data also exist for crustaceans (for an overview see Whitington, 1995). Although there are some differences, the insects and crustaceans also share similarities, which suggest a common developmental program for establishing the central axon pathways. The other two arthropod groups have been largely disregarded in this respect. Merely one representative in myriapods had been analysed by Whitington and co-workers (1995), which uncovered fundamental differences compared to insects and crustaceans. The pattern of early axonogenesis in *Ethmostigmus rubripes* bore no resemblance to these. However, the establishment of the ventral nerve cord in chelicerates remained elusive and was the objective of this thesis.

Before axonogenesis is even initiated it has been shown that the most striking difference in nervous system development between insects and crustaceans as a sister-group on the one hand and myriapods and chelicerates on the other lies in the formation of neural precursor cells. Instead of single NBs generated in the first two groups, which divide in a stem cell mode and give rise to unique cell lineages (Broadus and Doe, 1995; Doe, 1992; Skeath, 1999), in the latter whole groups of

cells are recruited for the neural cell fate that do not divide any further and invaginate as a compound (Chipman and Stollewerk, 2006; Dove and Stollewerk, 2003; Kadner and Stollewerk, 2004; Mittmann, 2002; Pioro and Stollewerk, 2006; Stollewerk et al., 2001). However, the molecular mechanisms underlying these processes are conserved in all four arthropod groups. Similar procedures are applied in recruiting cells to adopt the neural fate through the expression of proneural and neurogenic genes (Lehmann et al., 1981; Lehmann et al., 1983; Campos-Ortega and Hartenstein, 1997; Broadus and Doe, 1995; Cabrera et al., 1987; Dove and Stollewerk, 2003; Galant et al., 1998; Jimenez and Campos-Ortega, 1990; Kadner and Stollewerk, 2004; Martin-Bermudo et al., 1991; Mittmann, 2002; Pistillo et al., 2002; Romani et al., 1989; Skaer et al., 2002; Skeath and Carroll, 1992; Stollewerk, 2002; Stollewerk et al., 2001; Wheeler et al., 2003; Wulbeck and Simpson, 2002). The internalisation of neural precursor cells occurs in a sequential mode and also the number and arrangement of neural precursors within a hemineuromere is conserved (Döffinger and Stollewerk, 2010; Dove and Stollewerk, 2003; Kadner and Stollewerk, 2004; Stollewerk, 2002; Stollewerk and Simpson, 2005; Stollewerk et al., 2001). Studies conducted on anterior-posterior and dorso-ventral patterning have uncovered that several genes involved in the regionalisation of the *Drosophila* embryo show conserved expression patterns in chelicerates and myriapods (Chipman and Akam, 2008; Damen, 2002; Damen et al., 2005; Damen et al., 2000; Döffinger and Stollewerk, 2010; Dove and Stollewerk, 2003; Janssen et al., 2004). During fly neurogenesis these patterning genes are also known to confer spatial and temporal identity to the delaminating NBs, thus assigning a unique cell fate upon them (Bhat, 1996; Broadus et al., 1995; Doe, 1992; Goodman and Doe, 1993; Hartenstein and Campos-Ortega, 1984; Isshiki et al., 2001; McDonald et al., 1998; Skeath, 1999; Skeath and Carroll, 1992; Skeath and Carroll, 1994; Skeath and Doe, 1996; Truman and Bate, 1988; Urbach and Technau, 2003). This marker combination allows to readily identify the different NBs. Individual lineages in *Drosophila* were described in great detail including cell soma position and projection routes (Bossing et al., 1996b; Schmid et al., 1999) giving further insight into the development of the nervous system. Recent findings reveal that the mediation of spatial information to neural precursors seems to be at least partially conferrable in the groups of chelicerates and myriapods and genes involved in patterning processes execute dual functions during

development (Döffinger and Stollewerk, 2010; Dove and Stollewerk, 2003; Stollewerk et al., 2003).

4.1.1 The embryonic axon scaffold in spiders shows typical features of an arthropod nervous system and originates from segmentally organised neurons similar to insects and crustaceans

Data on neuropile architecture in spiders so far existed only for the adult, where the nervous system scaffold has been described in detail and comprises two parts. The supraoesophageal ganglion is composed of the actual brain, consisting of three neuromeres (proto-, deuto- and tritocerebrum), including the cheliceral neuromeres. The suboesophageal ganglion complex integrates the neuromeres of the pedipalps, the legs and the condensed abdominal nerve mass and comprises in *Cupiennius* 11 pairs of longitudinal tracts and 5 segmental commissures that connect left and right (summarised by Wegerhoff and Breidbach, 1995).

To clarify how the axon scaffold is formed during embryonic development and what it looks like in *Cupiennius salei* and *Achaearanea tepidariorum* was one intention of this work and was analysed in several significant developmental stages. My findings illustrate that in spiders, as a representative of the chelicerates, the embryonic ventral nervous system essentially resembles a typical arthropod nervous system. Even though inversion dramatically changes the morphology of the embryo during development as described before (Seitz, 1966; Foelix, 1996) a rope-ladder like ventral nerve cord is being established. In the embryo, in either germ band half a longitudinal tract interconnects the individual neuromeres in anterior-posterior direction. Commissural fibres reach across the midline to integrate left and right in both spider species. One commissure per segment is formed without separating into an anterior and posterior fibre tract during the entire embryonic development. In contrast to this observation, in *Drosophila*, although at first the anterior and posterior commissure are established in close proximity, they become separated by the migration of midline glial cells that ultimately come to lie in between the adjacent tracts (Hummel et al., 1999; Jacobs and Goodman, 1989a; Klämbt et al., 1991). As described previously, the ventral midline in spiders is of a transient nature and can be

characterised as epithelial tissue that does not give rise to any neurons or glial cells. With ventral closure at the end of embryonic development it becomes completely reduced. Consequently, there are no specific cells present in the midline that are capable of separating commissural tracts during axonogenesis. However, since in the adult spider five commissural tracts were identified separation must occur, possibly during larval stages. Furthermore, I could distinguish between two main nerve tracts per hemisegment that project laterally to innervate the periphery (SN and ISN). In insects such motor projections leave the CNS in a common bundle and separate as they enter the periphery (Jacobs and Goodman, 1989b; Thomas et al., 1984). In Crustaceans, the SN splits into two branches after innervating the appendages and an intersegmental nerve could be identified there as well (Brenneis and Richter, 2010; Vilpoux et al., 2006). In spiders I made a similar observation in that the SN tract bifurcates before reaching and innervating the legs, indicating a common ground pattern in arthropods.

Spider axonogenesis begins when cells of the NPGs, which are located in the more apical layers of the NE, differentiate and begin to form axonal projections. The first neurons specified leave the invagination groups, migrate towards the interior of the embryo and are positioned in medial areas while neural cells are still recruited to the sites apically (Stollewerk, 2004). I found that the establishment of the embryonic axon scaffold in *Cupiennius* and *Achaearanea* is taking place in the basal most layers of the NE and is based on segmentally organised neurons comparable to insects and crustaceans (Boyan et al., 1995; Duman-Scheel and Patel, 1999; Goodman and Doe, 1993; Thomas et al., 1984; Whitington, 1995). Axon growth and neuropile formation occur synchronously in prosomal segments that are equally advanced in development and follow the anterior-posterior gradient of development in opisthosomal segments. This is in contrast to *Ethmostigmus rubripes*, the only myriapod analysed with respect to axon pathfinding (Whitington et al., 1991). Primary longitudinal axonal tracts in this myriapod are established by neurons originating in the brain and projecting posterior, rather than by segmental neurons. As development proceeds these axons extend further along the longitudinal axis and become thicker. Axons originating from segmental neurons appear to contribute to the primary axonal tracts only at later stages. However, this finding could be a species specific trait and not representative for axonogenesis in myriapods in

general. In fact, in *Glomeris marginata*, another myriapod, I observed that following the generation of neural precursor groups, the neuromeres and the neuropile are established simultaneously in leg segments, which are equally advanced developmentally (not shown).

4.1.2 In spiders cell groups as well as single neurons pioneer axonal tracts

I have described here the general course of events of the formation of the embryonic axon scaffold. It was not possible to resolve these processes on the level of identified neurons, however, which is due to several facts. Data on neuron identity are basically elusive and specific markers to identify these are largely not existent. Moreover, markers to label individual axonal tracts are not yet available. Another factor hampering the detailed analysis of individual cells and their projection patterns is the fact that quite a large number of cells differentiate from the invagination sites at the same time and begin to form axonal projections. However, my results provide evidence that groups and single cells pioneer axonal tracts.

In many organisms the establishment of the major axon pathways in the CNS is generally primed by so-called pioneer neurons, early differentiating neurons that form distinct axonal projections in a stereotypic way, which is determined by cues from the neuroepithelium and interactions of growth cones during their differentiation. These axons in turn express certain molecular markers such as fasciclin II (Grenningloh et al., 1990), which function to guide later forming axons. Comparative analysis conducted on early differentiating neurons in insects and crustaceans identified a number of pioneer neurons, which were comparable in the position of the cell soma, the time of axonal outgrowth and axon morphology (Bastiani et al., 1986; Goodman et al., 1984; Goodman and Doe, 1993; Jacobs and Goodman, 1989a; Sink and Whitington, 1991; Thomas et al., 1984; Whitington et al., 1993b). A set of five neurons could be homologised based on these criteria and the expression of certain marker genes such as *even-skipped* (Fig. 4-1). These neurons were found to be responsible for the establishment of primary axon tracts pioneering the longitudinal and transverse axon bundles (Duman-Scheel and Patel, 1999; Thomas et al., 1984;

Whitington et al., 1993b). According to these findings, the longitudinal connectives in both groups are initialised by two neurons, namely vMP2 and pCC (nomenclature after *Drosophila melanogaster*) that project ipsilaterally and anteriorly. The aCC neuron and its crustacean homologue project towards the periphery to pioneer the intersegmental nerve. MP4, which is positioned medially, sends a projection anterior, pioneering the median fibre tract and when reaching the anterior commissure bifurcates to contribute to the anterior commissure. The axon of another neuron, RP2 initially projects along the fascicle already established by aCC on the ipsilateral side to the intersegmental nerve and then turns to pioneer its posterior root. Several other pioneer neurons were identified in insects and crustaceans that show similarities as well as differences between the groups and therefore could not be homologised (summarised by Whitington, 1995).

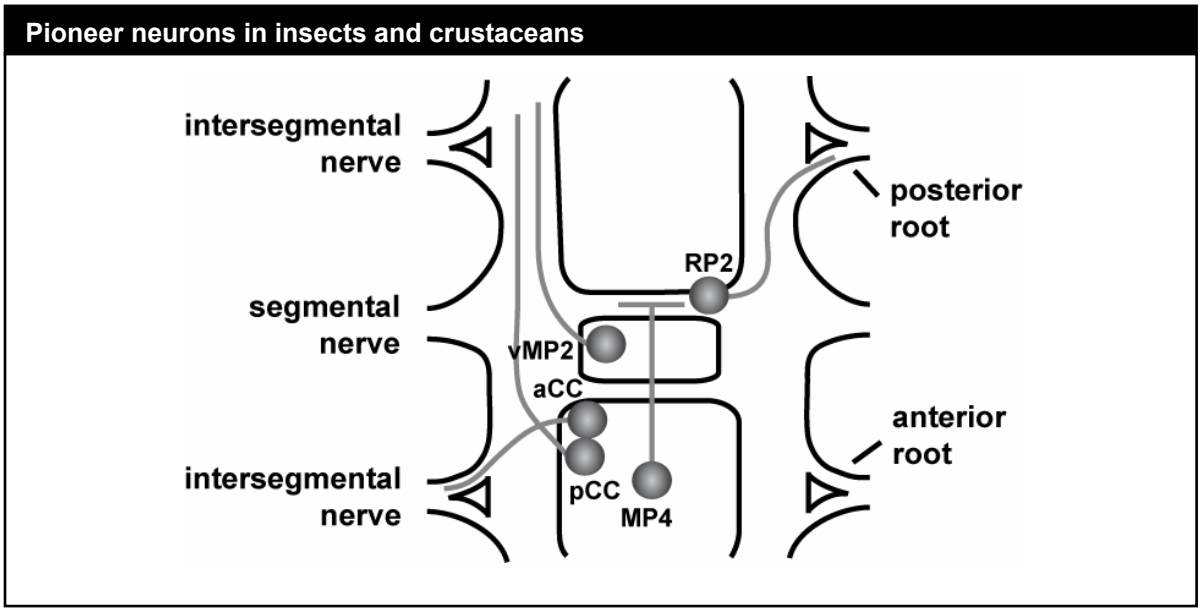

Figure 4-1: Common features of early axonogenesis in insects and crustaceans.

A set of five pioneer neurons could be homologised in insects and crustaceans that where shown to establish primary axonal tracts of the ventral nerve cord. Modified after Whitington, 1995.

Since the comparison of the formation of axon tracts during embryogenesis in *Cupiennius* and *Achaearanea* revealed substantial similarities between both spider species, the following assumptions for axonogenesis on a more cellular level are given only for *Achaearanea* and should account as representative for both. In *Achaearanea* at the beginning of axonogenesis I observed strong antibody staining against Tubulin in clusters of cells. The antibody specifically stains soma and axons

of neurons (for application in the nervous system see, Fischer et al., 2010; Harzsch et al., 1997; Mittmann and Scholtz, 2003). Expression in axons persists, while with ongoing maturation of nerve cells the staining in cell somata fades (LeDizet and Piperno, 1991; Piperno and Fuller, 1985). In prosomal hemisegments several of these clusters could be identified, which contained up to four cells. The solitary clusters observed in opisthosomal hemineuromeres consisted of up to 20 cells at about 80h of development (Fig. 4-2). The greater number of cells clustered in the abdomen probably reflects differences in maturation caused by the anterior-posterior developmental gradient. Initially prosomal clusters might have consisted of similar numbers of cells observable in the opisthosoma. Due to ongoing maturation, accumulation of Tubulin in cell bodies in the prosoma could have been reduced, while still strong in the abdomen. The first opisthosomal cell cluster located in the third abdominal segment was smaller than those in the remainder of the opisthosoma and could represent an intermediate developmental state between more anterior and more posterior lying segments. This would imply that expression of Tubulin is already weaker, but not as strongly down-regulated as in the prosoma. A different explanation could be tagma specific differences between the prosoma and the abdomen. Prosomal segments would then produce several small clusters, while in the abdomen a single large cluster is formed owing to different requirements of innervation. Such differences in the lineages produced by sequential NBs were observed between thorax and abdomen in the grasshopper (Bate, 1976a; Doe and Goodman, 1985b) and also in *Drosophila* segment-specific features of NB lineages were described (Bossing et al., 1996a; Bossing et al., 1996b; Prokop and Technau, 1994; Schmid et al., 1999; Truman and Bate, 1988). However, in *Achaearanea* the groups of cells in the prosoma and the opisthosoma were located in medial to lateral regions of the hemineuromere, exactly where the neuropile will be formed. The first axonal projections traceable within the hemineuromeres originate from these clusters, indicating that their neurons are in fact pioneer neurons that differentiate early to establish the primary trajectories of the developing embryo. From the arrangement of the clusters within the hemisegment one can conclude that each of them might stem from individual invaginations sites. Clonal analysis on *Drosophila* NBs of the VNC demonstrated, that the progeny of particular NBs are arranged in dense clusters and the location of these clones corresponds to the position of the parental neural precursor within the neuromere (Bossing et al., 1996b). Another

observation made, was that in general, cells of one cluster form distinct fascicles together. Furthermore it was shown that neurons of the same NB lineage in the developing fly brain often express the same genes and form developmental and also structural units. The somata of these cells remain close together and form clusters, their axons fasciculate to form the primary axonal tracts, which are the foundation of the *Drosophila* brain neuropile (Nassif et al., 1998; Younossi-Hartenstein et al., 2006). Indeed, I was able to produce similar findings in spiders. Although cells of the NPGs are probably not clonally related (Stollewerk et al., 2001), a functional or at least structural correlation between cells of one invagination group seems likely in *Achaearanea*. This suggestion was further supported by looking at older embryos.

With ongoing development a large number of differentiated neurons showed strong expression of Tubulin. These were also located in the medio-lateral part of the neuromere where before the pioneer clusters could be found. The axons of several neuronal cells lying close together were seen to fasciculate and form collective projections. This could be compared for example to motorneurons in *Drosophila* that derive from just a few NBs. Those that are morphologically similar were shown to be clonally related and innervate neighbouring muscles (Landgraf et al., 1997). Taken these observations into consideration, our findings suggest that also in the spider neurons differentiating from one NPG could be functionally related. Additionally to the centrally located bulk of neurons, anterior-medial in the hemisegment, Tubulin positive cells were positioned near the segment boundary whose axons also fasciculate. Possibly these cells correspond to neurons in insects that initiate the commissures or establish fascicles along which commissural axons can find their way to exit the neuropile.

Besides the grouped cells stemming from individual NBs, in some *Drosophila* NB lineages a few specific progeny cells migrate away from the main cluster to occupy positions further away from their siblings. Such cells, like aCC, pCC and RP2, project along separate routes and do not or only partially fasciculate with the other members of their clones (Bossing et al., 1996b). Furthermore, several of these neurons are described to pioneer tracts in the developing nervous system as illustrated above and are possibly conserved in insects and crustaceans (Duman-Scheel and Patel, 1999; Thomas et al., 1984; Whitington et al., 1993b; Whitington, 1995). Interestingly, apart

from the clusters of pioneer neurons in *Achaearanea*, in opisthosomal segments I could identify an additional solitary neuron, which lies aside from the large cluster in a more anterior-medial position. This neuron shows a remarkable, very distinct axonal projection. Initially it projects towards the segmental boundary and then, almost upon reaching it, turns laterally to elongate alongside it and projects towards the area where the intersegmental nerve will be formed (Fig. 4-2). Based on the position within the neuromere and the projection pattern, this neuron might be comparable to RP2, which was shown to pioneer ipsilateral connectives by originating close to the anterior commissure and projecting anterior before it turns to initiate the posterior root of the ISN (Doe et al., 1988; Thomas et al., 1984). Observations obtained in *Cupiennius* further support the assumption that indeed the identified pioneer neuron is homologous to insects and crustaceans. In *Cupiennius* two invagination groups were detected that generate *even-skipped* positive neurons. These were the NPGs b3 and b4, which are located in the anterior medial area of the hemisegment (Döffinger and Stollewerk, 2010). The number and positions of NPGs in *Cupiennius* and *Achaearanea* are almost perfectly identical (Döffinger and Stollewerk, 2010), which led me to assume that from the position within the hemineuromere this solitary pioneer neuron could stem from either of the two neural precursor groups. In both insects and crustaceans the RP2 neuron expresses *even-skipped*, which was one of the conditions the homology of pioneer neurons was based upon (Duman-Scheel and Patel, 1999; Thomas et al., 1984; Whitington et al., 1993b). In the prosoma of *Achaearanea* embryos this neuron could not be identified, but this is possibly due to the large number of Tubulin positive neurons, which made it difficult to distinguish individual cells. Nevertheless, these findings are a first hint that in chelicerates the embryonic axon scaffold might be established by neurons that are similar to the insect and crustacean counterparts and thus support evolutionary conservation of axonogenesis in arthropods.

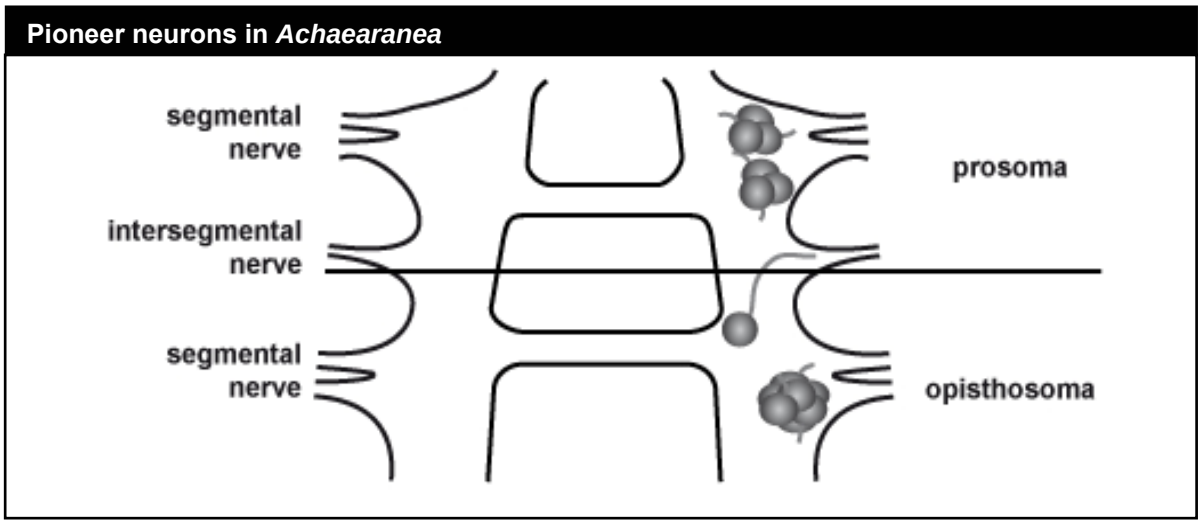

Figure 4-2: Early axonogenesis in *Achaearanea tepidariorum.*

The panel shows pioneer neurons, which are involved in the establishment of primary axonal tracts in the spider *Achaearanea tepidariorum*. Single cells as well as whole cell groups function to pioneer the embryonic axon scaffold. The horizontal line distinguishes the prosoma from the opisthosoma.

To summarise, in spiders axonal tracts are established by segmentally organised neurons, a characteristic which can be considered a conserved feature throughout the arthropod phyla. Similar to insects and crustaceans, I found whole clusters of cells that seem to function as pioneer neurons as well as single cells, which form the primary axonal tracts.

4.2 The vertebrate floor plate and ventral midline in spiders share morphological characteristics

The CNS of higher organisms is bilateral symmetric since they must be able to integrate sensory input and coordinate motor control on both sides of the body. Thus information must be transferred between the two sides, which is realised through commissures formed by neurons that project their axons across the midline to the contralateral side of the CNS. Other neurons extend but never cross the midline, rather they project ipsilaterally. Across the animal kingdom the ventral midline constitutes an important choice point for pathfinding axons. In both insects and vertebrates the two symmetrical body halves are separated by a specialised group of cells, which in *Drosophila* form the ventral midline and in vertebrates the floor plate (Jacobs and Goodman, 1989a; Jessell et al., 1989; Klämbt et al., 1991). Midline cells are the first cells to differentiate during development (Crews et al., 1988; Schoenwolf

and Smith, 1990; Thomas et al., 1988). In vertebrates the floor plate is of a transient nature, whose cells are eliminated by apoptosis as soon as their role in nervous system development is fulfilled (Hankin et al., 1988; Knyihar-Csillik et al., 1995; Laywell and Steindler, 1991) and by the end of embryonic development the entire floor plate is disposed of. Similar observations came from studies in *Drosophila*, where specific midline cells with a morphogenetic function undergo programmed cell death once their mission is accomplished (Sonnenfeld and Jacobs, 1995). Apart from their role in axon pathfinding midline cells also exert a number of other functions. In the vertebrate neural tube, the floor plate cells contribute to dorso-ventral patterning of the CNS (Placzek et al., 1991; Yamada et al., 1991) and are involved in the specification of different neuronal and glial cell populations (Briscoe and Ericson, 1999; Briscoe and Ericson, 2001; Ericson et al., 1996; Orentas and Miller, 1996; Poncet et al., 1996; Pringle et al., 1996). *Drosophila* midline cells additionally control the patterning of the ventral ectoderm (Golembo et al., 1996; Kim and Crews, 1993; Schweitzer et al., 1995) and induce the formation of specific mesodermal cells (Lewis and Crews, 1994; Luer et al., 1997).

In *Drosophila*, cells of the ventral midline have such distinct properties, that they were named mesectoderm to distinguish them from the adjacent mesoderm and the neuroepithelium and constitute a boundary between those two germ layers (Poulson, 1950). After gastrulation they maintain transient adhesive contact to the mesoderm and morphologically look like mesodermal cells rather than the neuroectodermal cells immediately next to them. Eventually they loose contact to the mesoderm but can still be discerned from the neighbouring neuroepithelium (Foe, 1989). Finally, after differentiation they comprise a set of about 20 uniquely identifiable cells per segment, consisting of both neurons and glial cells (Bossing and Technau, 1994; Jacobs and Goodman, 1989a; Klämbt et al., 1991). Differently from *Drosophila* the vertebrate floor plate does not produce neurons (McKanna, 1993). Instead, it is composed of specialised columnar glia-like (ependymal) cells, that are also morphologically distinct from neighbouring tissues (reviewed by Colamarino and Tessier-Lavigne, 1995). The spatially restricted expression of several molecular markers indicates that the floor plate is composed of several different groups of cells even though all of them were characterised as glial cells. Thus along the medio-lateral axis the floor plate can be

partitioned into medial (MFP) and lateral floor plate (LFP) (reviewed by Placzek and Briscoe, 1996).

The ventral midline in spiders, also termed ventral sulcus (Anderson, 1973), was described as a single layered epithelium separating the left and right germ band halves. As Seitz observed (1966) the midline or ventro-median furrow is generated from cells of both germ band halves. The first appearance of this tissue is noted at the onset of inversion, when the germ band halves are pushed away from each other by the widening of the midline epithelium. During inversion the epithelium is spanning the space between both migrating germ band halves continuously from the beginning of the morphogenetic movement through the entire embryonic development, but becomes completely reduced when embryogenesis ends. That the spider midline is of a transient nature, which does not give rise to any neural cells, is supported by observations made in our group and by others. No neural markers such as the proneural genes *CsASH1*/*2*, the neural cell fate determinant Prospero and the differentiation markers *islet* and *even-skipped* were found to be expressed in the spider midline (Döffinger and Stollewerk, 2010; Stollewerk, 2002; Stollewerk, 2004; Stollewerk et al., 2003; Stollewerk et al., 2001; Weller and Tautz, 2003). Initially, before the onset of inversion cells of the ventral midline epithelium are indistinguishable from the adjacent neuroepithelium as was reported by Seitz for *Cupiennius salei* (1966) and by Akiyama-Oda and co-workers for *Achaearanea tepidariorum* (2006). However, in *Achaearanea* this area was shown to be characterised by the expression of *fork head* (*fkh*), *short gastrulation* (*sog*) and *single minded* (*sim*) in nearly the same cell population. With beginning inversion, though, alternative patterns of gene expression emerge. The transcripts of *fkh* and *sog* no longer show an overlap with the expression of *sim*, which might reflect the differentiation of several cell types within the ventral region. Expression of *fkh* is lost from the medial most cells and the *sog* domain splits into two lines adjacent to the *sim* expressing region (Akiyama-Oda and Oda, 2006). Floor plate cells in vertebrates express members of the *forkhead* gene family of transcription factors (Charrier et al., 1999; Odenthal and Nusslein-Volhard, 1998; Odenthal et al., 2000; Strahle et al., 1993; Strahle et al., 1996), which were shown to characterise ventral midline cells and to be required for their formation (Ang and Rossant, 1994; Echelard et al., 1993; Ruiz i Altaba et al., 1993; Weinstein et al., 1994).

4.3 In spiders *single minded* does not play a role in midline formation

The correct development of all CNS midline cells in *Drosophila* depends on the function of the midline master regulatory gene *sim*. In *sim* mutants the midline cells are not specified correctly, in fact neuronal and non-neuronal precursor cells die after gastrulation resulting in the improper formation of the entire axon scaffold (Nambu et al., 1990; Thomas et al., 1988). The first expression of *sim* transcript in *Drosophila* can be observed in two rows of midline progenitors of the blastoderm embryo at cellularisation. After gastrulation sim protein is localised in cell nuclei along the entire axis of the embryonic ventral midline. Initially the transcription factor is present in all midline progenitors but after their differentiation, expression of *sim* is restricted to midline glial cells, where it is maintained throughout embryonic development (Crews et al., 1988; Thomas et al., 1988). Besides specifying midline cell fate, *sim* contributes to axon pathfinding and ventral patterning via the regulation of a number of CNS midline genes. These genes resume the developmental program of midline CNS development, which includes neuronal and glial cell formation, axon guidance, and functional differentiation (Nambu et al., 1990). In addition to its expression in the ventral nervous system, *sim* can be found in a subset of ventral muscle precursors, the gut as well as in the brain where it might play a role in regionalisation and cell fate determination (Crews et al., 1988; Lewis and Crews, 1994).

In spiders *sim* was first cloned in *Achaearanea* by Hiroki Oda's group (2006) and shown to be expressed in a ventral median stripe along the germ band. It was part of this thesis to re-examine *sim* expression over the course of embryogenesis with respect to the spider ventral midline. In *Achaearanea* I observed expression of *sim* in a longitudinal band along the ventral side of the embryo when the germ band is fully extended and neurogenesis begins. The expression pattern included all prosomal segments with the exception of the cephalic region and the entire segmented opisthosoma. This pattern looked reminiscent to that in the fly at a comparable developmental time point, immediately after gastrulation and before individual midline cells are specified. However, higher magnifications confirmed that the *sim* expressing cells in *Achaearanea* morphologically could not be discerned from cells of the neighbouring NE, where the gene was not expressed. This is different from

Drosophila, where the *sim* positive midline precursors are clearly distinguishable from the adjacent neuroectodermal tissue (Foe, 1989). Analysis with Cadherin, which visualises neural precursor groups, revealed that the *sim* expressing cells are not cells of the actual NE as no invagination sites are formed in the area of *sim* expression. Rather, the stripe of *sim* positive cells lies immediately adjacent to the neurogenic region. With the beginning of inversion and the emergence of the ventral midline in spiders, *sim* expression persisted in the medial most regions of the germ band halves but was excluded from the midline epithelium stretching between them. The cells of this epithelium in turn were clearly different in morphology from both the NE as well as the *sim* positive cells as described above and existed only transiently.

Two transcription factor homologues (*Sim1* and *Sim2*) have been identified in several vertebrate species, which show high similarities to the conserved domains of the *Drosophila sim* gene. Their expression patterns were shown to be largely reminiscent of those in the fly. Expression was found in the developing brain, the ventral spinal cord, muscles, the gut and in excretory organs (Coumailleau et al., 2000; Eaton and Glasgow, 2006; Ema et al., 1996; Fan et al., 1996; Moffett et al., 1996; Pourquié et al., 1996; Wen et al., 2002), where *sim* is involved in the regionalisation of these tissues. However, whereas in *Drosophila sim* is the key regulator for midline cell development, vertebrate *sim*, although found close to the ventral midline region of the spinal cord is not expressed in floor plate cells. Mutations in the vertebrate genes lead to defects in various organs, including the nervous system (Eaton and Glasgow, 2006; Goshu et al., 2002; Michaud et al., 1998; Shamblott et al., 2002), but requirement in floor plate specification similar to *Drosophila* could not be demonstrated for vertebrate *sim*, nor does is seem to be involved in axon pathfinding.

With ongoing inversion in *Achaearanea sim* expression was gradually restricted to clusters of cells that were found in medial to basal layers of each hemineuromere. Also these cells resided somewhat separate as if, within the neuromere, the *sim* expressing cells formed a compartment on their own, providing further evidence that these cells indeed differ from the neuroectodermal tissue. If one assumes, that the *sim* expressing cells do not belong to the ventral midline epithelium either, which is supported by the fact that *sim* expression was never observed in this tissue, then the mid-embryonic expression pattern of *sim* could be comparable to vertebrates. In mice

for example *Sim1* is expressed in cell clusters immediately adjacent to the lateral floor plate but never in the floor plate itself (Ema et al., 1996; Fan et al., 1996). Although early expression of *sim* was somewhat similar to *Drosophila* in appearance, the later observed expression pattern and the transient epithelial structure of the spider ventral midline suggest that in spiders *sim* is not involved in regulating midline cell fate. To verify this assumption embryonic RNA interference experiments were conducted in *Achaearanea* to knock-down gene function. As mentioned above, in *Drosophila* loss of *sim* function results in misspecification and cell death of ventral midline progenitors, which in turn leads to severe phenotypes concerning ventral patterning and axon pathfinding (Nambu et al., 1990; Thomas et al., 1988). In *Achaearanea* I could not find supportive data for a similar role of *sim*, which was already suggested by the expression data obtained. After down-regulation of the gene transcript, embryonic development was not impaired with respect to the ventral midline. The process of inversion that involves major reorganisations of the embryo appeared to proceed in a normal way. In general *sim* depleted embryos developed entirely normal. Since I was not able to analyse the injected embryos in greater detail using immunohistochemical approaches, these findings can only give a glimpse of *sim* function in spiders and should be regarded as preliminary.

Additionally to the expression of *sim* in the ventral most region of the spider embryo, expression of the gene was also observed in the developing cephalic lobes and in non-neural tissues such as the bases of the appendages. Findings in vertebrates support a role for *Sim* in the patterning of these areas. It was shown for example that in zebrafish *Sim1* is required for cell development in the forebrain and the hypothalamus (Eaton and Glasgow, 2006; Goshu et al., 2002) and expression of both *Sim1* and *Sim2* is related to different steps of limb myogenesis in chick and mice (Coumailleau and Duprez, 2009). In the spider *sim* might be involved in patterning processes comparable to those in vertebrates requiring *sim* signalling.

4.4 *Netrin* in spiders plays a role in different neurogenic events

4.4.1 The function of *netrin* in axon pathfinding and commissural guidance is conserved in spiders

Much evidence is provided that from worms and flies to rats and humans a common mechanism determines which axons cross the midline and which do not (reviewed by Dickson, 2002). Many CNS axons initially grow towards the midline and then either turn longitudinally without crossing (ipsilateral), or by first crossing the midline and then turning (contralateral). Attractive signals guide commissural axons over sometimes long distances to the nervous system midline. Furthermore, short-range interactions between guidance cues provided by midline cells and their receptors expressed by pathfinding axons, allow commissural axons to cross the midline only once and prevent ipsilateral axons from crossing. The molecules involved in axon pathfinding are highly conserved across species. Among these factors are the Netrins, secreted molecules, which form a concentration gradient that can be interpreted by navigating growth cones. These proteins function in a dual way in that they are chemoattractive for some neurons while chemorepellent for others and carry out their functions by interacting with specific receptors (reviewed by Barallobre et al., 2005; Livesey, 1999; Kaprielian, 2001). Netrins are expressed in midline cells in all animals analysed. In *Drosophila* two Netrins, NetA and NetB were described as guidance cues (Harris et al., 1996; Mitchell et al., 1996) that are expressed in the ventral midline where they communicate an attractive signal for commissural axons (Brankatschk and Dickson, 2006; Kolodziej et al., 1996) as well as repulsive cues (Keleman and Dickson, 2001; Labrador et al., 2005). Vertebrate *netrin-1* is expressed by floor plate cells and promotes outgrowth and guidance of commissural axons towards the floor plate (Kennedy et al., 1994; Serafini et al., 1996).

The process of inversion in spiders as described above represents an obstacle to overcome during axon pathfinding. While the ventral midline epithelium expands to cover the entire width between the migrating germ band halves, it must be ensured that outgrowing and crossing commissural projections receive continuous guidance information to correctly connect both sides of the embryo. Even so, the guidance of

commissural axons towards and across the midline seems to be facilitated by Netrin in a conserved mode similar to insects and vertebrates. I could show that in both spider species analysed in the NE *netrin* was expressed in the region of commissural outgrowth and contralateral projecting axons pointed in the direction of the *netrin* expressing cells. In addition to this, *netrin* was also expressed in transverse stripes in the ventral midline and this expression was maintained throughout the entire process of inversion, so that the bands of *netrin* positive cells expanded continuously during this morphogenetic process and retracted again after dorsal closure. Therefore, these cells quite possibly function as a permanent source of *netrin* signal to provide guidance information for outgrowing axonal projections throughout embryogenesis. In *Drosophila* at first *netrin* expression is expressed equally distributed in all midline cells but then becomes restricted to a certain population of cells, namely the midline glia (Mitchell et al., 1996). However, the spider ventral midline does not give rise to any specific cell types such as neurons and glia but *netrin* is expressed in epithelial cells. The epithelial cells that express *netrin* cannot be distinguished from the *netrin* negative cells of the midline. The vertebrate floor plate consists only of glial-like cells. Nevertheless, as the expression data for a number of molecular markers revealed, it is composed of several different groups of cells (reviewed by Placzek and Briscoe, 2005) indicating that although similar in morphology different cellular domains can be defined within the floor plate. Besides its cellular expression, I detected *netrin* transcripts in the commissural axons during their outgrowth and extension across the midline. This has not been described in other species. Analysis of *netrin* expression in *Drosophila* confirmed that *netrin* transcripts are not present in commissural axons (not shown).

To support the expression data obtained and analyse *netrin* function in spiders, RNAi experiments were conducted. In *Cupiennius* embryos treated with ds *netrin* RNA I observed that the formation of commissures, longitudinal connectives and peripheral projections was impaired. Although, in general axonal projections are formed, fewer of them actually exit the hemineuromere to reach and cross the midline so that the commissural axon tracts appeared to be thinner. Longitudinal tracts displayed breaks in their course and failed to connect adjacent neuromeres in a correct manner. If no *netrin* is present at the time of commissural outgrowth, axons normally attracted by the possible long-range function of the guidance cue located in the region of

commissural outgrowth might grow in any direction. Those axons that do extend towards the midline and actually leave the NE have passed by the area where *netrin* is normally provided and now fail to recognise further guidance information since cells of the ventral midline epithelium also lack *netrin* signal. Both observations are in accordance with findings in *Drosophila* and vertebrates, where the absence of *netrin* leads to defects in axon pathfinding and commissure formation. In *Drosophila* deletion of both genes results in thinner commissural projections, indicating that fewer axons have crossed the midline, as well as in breaks in the longitudinal connectives (Harris et al., 1996; Mitchell et al., 1996). In homozygous *netrin-1* mutant mice, commissural axon trajectories are severely affected. Most commissural axons are shorter than normal and although growing ventro-medially at first only a few projections actually reach the floor plate to cross to the contralateral side (Serafini et al., 1996). That the longitudinal tracts are not formed properly is more difficult to interpret, since *netrin* is not expressed in the area where these fascicles are formed. However, recent findings in *Drosophila* hint at a function of Netrin in glial migration and *netrin* mutants show longitudinal and ISN phenotypes, which are partially due to glial cell migration defects (von Hilchen et al., 2010).

In addition to midline guidance, Netrins were shown to also influence peripheral projections. To facilitate this function in *Drosophila netrins* are expressed by discrete subsets of muscles. In *netrin* double mutants axons fail to correctly innervate the periphery. It was shown that the intersegmental nerve, although early projections are formed normally, later branches inappropriately, stalls or extends past its correct target muscles (Labrador et al., 2005; Mitchell et al., 1996). In spiders I could show that *netrin* is expressed in cells at the base of the pedipalps and the walking legs. In RNAi treated embryos this expression of *netrin* was absent, which led to a slightly improper formation of the peripheral trajectories, especially the ISN. Possibly in spiders guidance of peripheral axon tracts is organised in a similar way to *Drosophila*. In any case, it seems that *netrin* expression is restored to some extend during later development and therefore some late projecting axons might project towards the correct area and partially rebuilt axonal tracts.

The observed expression pattern and the malformations during axon pathfinding and commissure formation caused by depletion of *netrin* suggest that in spiders this

function of *netrin* is conserved, similar to vertebrates and *Drosophila*. My findings also illustrate that expression of *netrin* was adapted to the specific morphology of the spider embryo to fulfil its requirement in axonogenesis.

4.4.2 *Netrin* is involved in neural differentiation of *Cupiennius salei*

Besides its conserved function in axon pathfinding and commissural guidance, a different possible concept for *netrin* in developmental processes has emerged during the course of this work. During early neurogenesis *netrin* is strongly expressed in a ring-like pattern surrounding the invagination sites of the NE in *Cupiennius* as was described before. In cross-sections it became obvious that the *netrin* expressing cells ensheath neural precursor groups separating individual invagination sites from one another. These sheath cells were shown to display typical morphological characteristics of glial cells. Furthermore, a large part of the surrounded neural precursor groups did not differentiate until larval stages and cells within the groups maintained their epithelial character (Stollewerk, 2004). Besides multiple other functions glial cells both in vertebrates and invertebrates were proposed to play important roles in the formation and maintenance of neuropile compartments (reviewed by Steindler, 1993). In *Drosophila* compartmentalisation of neural structures is observed in the larval brain where neuroblast lineages are ensheathed by glial septa that first appear in the embryo and remain visible throughout postembryonic development (Younossi-Hartenstein et al., 2003). Delayed differentiation of neural precursors as observed in *Cupiennius* in the precursor groups is also not an uncommon feature displayed in insects. It has been reported that larval NBs in the fly are generated already during embryonic development but are then arrested in an immature state until they enter the cell cycle again during larval stages (Ito and Hotta, 1992; Truman and Bate, 1988). These NBs and their progeny do not finish their differentiation until metamorphosis and were shown to be enclosed by glial sheaths comparable to the situation in the spider (Prokop and Technau, 1991; Truman and Bate, 1988). Postembryonic NBs were also identified in the Tobacco hornworm, *Manduca sexta* where they are thought to play a major role in contributing new neurons to the segmental nervous system (Booker and Truman, 1987).

Functional analysis in *Cupiennius* embryos using the embryonic RNAi approach revealed that when the expression of *netrin* in the sheath cells surrounding the invagination sites was reduced, the morphology of neural precursor groups was altered. I assume that loss of *netrin* led to premature specification of neural cells, resulting in larger numbers of cells expressing the differentiation marker *islet*. Possibly this premature differentiation is at the expense of other cells, which would normally remain epithelial until a later developmental time point and adopt different cell fates. These findings hint at a function of *netrin* in spiders, which has not yet been described in other organisms. Considering this aspect *netrin* could be involved in maintaining the epithelial structure of the invagination groups and thereby delaying differentiation of neural precursor cells until later developmental stages. Similar to *Drosophila*, these neural precursor cells could, after a dormant phase be re-activated and would then differentiate to contribute to larval or even adult neuronal structures.

Although for other organisms no data exist that suggest an explicit role of *netrin* in the differentiation of neural precursor cells, recent studies propose the involvement of *netrin*, independent of its function as guidance factor, in other processes. As described before, *netrin* expression is not only restricted to the nervous system, indicating a function in morphogenetic processes of multiple organs. While most classical studies were based on a function of *netrin* as a long-range guidance cue, data are accumulating providing evidence that *netrins* can also function at short distances. In vertebrates Netrins are speculated to be involved in epithelial morphogenesis for example of the mammary gland, where *netrin-1* exerts adhesive function to stabilize a progenitor cell layer mediated by its receptor Neogenin (Srinivasan et al., 2003). A similar requirement of Neogenin was observed in zebrafish somitogenesis, although it is not clear whether *netrin-1* is also the ligand in this process (Mawdsley et al., 2004). Another organ that involves *netrin* dependent signalling is the lung, where outgrowth and fine-tuning of the size and shape of emerging epithelial buds is dependent on *netrin* (Dalvin et al., 2003; Liu et al., 2004). In the pancreas *netrin-1* operates as adhesive and migratory cue for epithelial pancreatic progenitors (Yebra et al., 2003) and during angiogenesis it fulfils various roles involving different receptors (Lu et al., 2004; Nguyen and Cai, 2006; Park et al., 2004). These recent insights into multiple short-range functions of *netrin* in non-neural tissues invoke the question of whether similar roles exist in the nervous

system. And indeed, in vertebrates it was also shown that *netrin-1* accumulates in the paraxial space of the spinal cord in adult rats and is expressed in both neurons and glial cells, suggesting a function in the maintenance of neuron-glial interactions (Manitt et al., 2001). In *Drosophila netrin* acts as short-range cue in regulating the development of neuromuscular synapses (Mitchell et al., 1996; Winberg et al., 1998). Also in commissure formation the guidance across the ventral midline is thought to be achieved via a short-range action of *netrin* (Brankatschk and Dickson, 2006). This might be also the case in spiders, where *netrin* signal is continuously provided by midline cells to guide axons to the contralateral side. In the context of cancer Netrins were proposed to participate in cell survival and apoptosis via their receptors DCC and UNC 5 (Fitamant et al., 2008; Llambi et al., 2001; Mazelin et al., 2004; Mehlen et al., 1998), a function that was shown recently to apply to the nervous system as well (Furne et al., 2008).

The broad scientific approach into functions of *netrin* other than as a guidance factor illustrates the involvement of a single molecule in various developmental processes. The above illustrated role of *netrin* exerted in *Cupiennius* might be related to the involvement in cell adhesion and cell-cell interactions as it was proposed by several groups in vertebrates.

5 Summary

Neurogenesis and axon pathfinding have been subject to a great deal of scientific projects in various organisms over the last decades and the amounting data in insects and crustaceans give a good overview on how a nervous system is established in arthropods. However, although the developmental processes are well understood in these groups, research in chelicerates and myriapods in a similar direction has been neglected. The main focus of the present work therefore was to uncover the mechanisms involved in axon pathfinding and midline guidance in two representatives of the chelicerates, the spiders *Cupiennius salei* and *Achaearanea tepidariorum*. A prerequisite for the detailed analysis of these processes is the knowledge of CNS architecture. Hence, in a first step I described the morphology of the nervous system throughout embryonic development and was able to show that in spiders essentially a typical arthropod nerve cord is being established. Furthermore, the spider axon scaffold is formed on the basis of segmentally organised neurons of which groups as well as single cells pioneer axonal tracts. Specifically, I could identify an individual neuron, which in position, projection pattern and marker expression is comparable to the RP2 neuron in *Drosophila* that pioneers the posterior root of the segmental nerve.

In a second step I analysed the spider ventral midline with respect to its possible function in axonal guidance. It was shown that the ventral midline epithelium, which spans the gap between the germ band halves during the entire process of inversion, is of a transient nature and does not give rise to neural cells. It is thus comparable to the floor plate of vertebrates, which also only exists transiently. The examination of *single minded* revealed that different to *Drosophila*, where *sim* is expressed in midline cells and functions as the key regulatory gene for midline specification, in spiders *sim* is not expressed in the midline epithelium but adjacent to it and the NE, comparable to vertebrate Sim. Furthermore, I observed *sim* expression in the bases of the appendages and in the head. Like in vertebrates it might be involved in pattering processes of these tissues. Nevertheless, the midline in spiders functions as an

organising centre for outgrowing commissural axons, a function it shares with other invertebrates and vertebrates.

The signalling network involved in axon pathfinding at the midline is highly conserved across species. In this work I was able to identify one *netrin* homologue in both *Achaearanea* and *Cupiennius* and could provide evidence for a conserved function of the chemotropic guidance factor in commissural axon guidance. In RNAi experiments that down-regulated *netrin* function, the formation of the entire embryonic nerve cord, in particular that of the commissures, was severely affected. Furthermore, I could identify a novel function for *netrin*, which has not yet been described in other organisms. Besides its role in axon pathfinding *netrin* appears to be involved in epithelial morphogenesis in the central nervous system. In this function *netrin* is required in glial cells that surround the epithelial vesicles of the neural precursor groups to maintain the undifferentiated state of neural precursors. Depletion of *netrin* leads to the premature segregation of neural precursor cells from the invagination sites and to an increase of cells that express the early differentiation marker *islet*.

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7 Appendix

I Abbreviations

α	anti
aa	amino acid
aCC	anterior corner cell
AP	anterior-posterior
AP-staining	alkaline phosphatase staining
ASH	*achaete-scute* homologue
cDNA	complementary DNA
ch	chelicerae
CNS	central nervous system
Cy5	indodicarbocyanine
DEPC	diethyl carbonate
DIG	digoxigenin
DNA	deoxyribonucleic acid
ds	double stranded
DV	dorso-ventral
EB	elution buffer
EDTA	ethylenediaminetetraacetic acid
et al.	and others
EtBr	ethidium bromide
EtOH	ethanol
FITC	fluorescein
h	hour/hours
g	gram
GMC	ganglion mother cell
kb	kilo base
l	leg segment
LSM	confocal laser scanning microscope
M	Molar
MeOH	methanol
mg	milligram

min	minute
ml	midline
ml	millilitre
mM	millimolar
mRNA	messenger RNA
µg	microgram
µl	microlitre
n	number
NB	neuroblast
NE	neuroectoderm
NPG	neural precursor group
pNPG	primary neural precursor group
sNPG	secondary neural precursor group
o	opisthosomal segment
ON	over night
pCC	posterior corner cell
PCR	polymerase chain reaction
ped	pedipalp
ph	potential hydrogenii
PNS	peripheral nervous system
RNA	ribonucleic acid
RNAi	RNA interference
rpm	rounds per minute
RT	room temperature
sec	seconds
sim	*single minded*
Tab.	Table
VNC	ventral nerve cord
VNE	ventral neuroectoderm

II Solutions, chemicals and reagents

Solutions

Aqua dest.

Deionised autoclaved H_2O.

Chloroform-Isoamylalkohol

Chloroform and Isoamylalkohol are mixed in a 28:1 ratio and stored at 4°C.

DEPC-H_2O

0,001% DEPC autoclaved in Aqua dest.

Detection buffer for alkaline phosphatase

0.1M NaCl

50mM $MgCl_2$

50mM Tris-HCl

0.1% Tween20

pH 9,5

Electrophoreses gel

0.5%: 0,25g agarose in 50ml 1xTAE, heated in the microwave.

2%: 1g agarose in 50ml 1xTAE.

Fixative for immunohistochemistry and phalloidin

50% (v/v) n-heptane

50% (v/v) 5.5% MeOH-free formaldehyde in PEMS or PBS.

Fixative for *in situ* hybridisation

50% (v/v) n-heptane

50% (v/v) 5.5% formaldehyde in PEMS or PBS.

hyb A (5ml)

50% (v/v) formamide

5x SSC, pH 5.5

2% (v/v) 10mg/ml boiled sonicated salmon testis DNA (ssDNA)

0.5% (v/v) 20mg/ml tRNA

0.05% (v/v) 100mg/ml heparin stock

DEPC-H_2O is added to a total volume of 5ml.

Solution should be prepared freshly before using.

hyb B (50ml)

50% (v/v) formamide

5x SSC, pH 5.5

0.1% Tween 20

DEPC-H_2O is added to a total volume of 50 ml.

Solution can be stored at -20°C.

LB-(Luria Bertani) medium

10g Bacto-Trypton

5g Bacto-yeast extract

10g NaCl

dissolve components in 900µl aqua dest., adjust pH to 7 with 5N NaOH and fill up to 1 litre, autoclave and store at 4°C.

Loading buffer for RNA gels

50 % (v/v) formamide

5% formaledhyde (37%)

2.5% (v/v) ethidium bromide (10mg/ml)

1% (v/v) DEPC-H2O

1x MOPS

1% (v/v) RNA sample

10x MOPS Stock solution

0,2 M MOPS (pH 7)

50 mM sodium acetate (pH 7)

10 mM EDTA (pH 8)

NBT/BCIP staining solution

1ml alkaline detection buffer

3,5µl BCIP (5-bromo-4-chloro-3-indolyl-phosphate), 50mg/ml

4,5µl NBT (nitroblue-tetrazoliumchloride), 50mg/ml in 70% DMF (N,N-dimethylformamide)

20xPBS (phosphate buffered saline) stock solution

1.3M NaCl

0.07M Na_2HPO_4

0.03M NaH_2PO_4

set to pH 7,4

1xPBS working solution (referred to as PBS)

dilute 20xPBS to a ratio of 1:19

1xPBT working solution (referred to as PBT)

1xPBS with 0.3% Triton-X-100

1xPBTween working solution (referred to as PBTw)

1xPBS with 0.1% Tween (for *in situ* hybridisation)

1xPBS with 0.02% Tween (for Phalloidin and antibody staining)

PEMS (PIPES, EGTA, $MgSo_4$)

100mM PIPES

1mM EGTA

2 mM $MgSO_4$

pH 6,9

Phenol/Chloroform

1 vol phenol, 1 vol chloroform

Phenol-chloroforme-isoamylalcohol

Phenol, chloroform and isoamylalcohol mixed in a ratio of 25:24:1 and stored at 4°C.

50xTAE (Tris-Acetat-EDTA) stock solution

242g Tris are dissolved in 500ml aqua dest.

100ml 0.5M Na2EDTA, pH 8

57.1ml glacial acetic acid

Aqua dest. is added to a total volume of 1 litre.

1xTAE working solution (referred to as TAE)

dilute 50xTAE to a ratio of 1:49

Chemicals and reagents

Acetic anhydride (Fisher)

Agarose (Seakern)

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

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

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

$CaCl_2$

Chlorine bleach (Vortex Thick Bleach)

Chloroform (VWR)

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

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

DTT (0.1 M, Dithiothreitol) (Invitrogen)

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

Ethanol (EtOH)

EDTA (Ethylenediaminetetraacetic acid) (VWR)

Ethidium bromide

Formamide (Sigma)

Formaldehyde (37%) (VWR)

Formaldehyde (MeOH-free) (Polyscience Inc)

Glycerol (VWR)

n-Heptane (VWR)

Isopropanol

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

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

LiCl (Fisher)

$MgCl_2$ (VWR)

Methanol (MeOH)

Mouse serum (Sigma)

NBT (Nitro blue tetrazolium chloride) (Sigma)

Phalloidin (Invitrogen)

Phenol Chloroform (Sigma)

Proteinase K (41 u/mg) (Sigma)

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

RNAse H (2 /µl) (Invitrogen)

RNAse-Inhibitor (40 u/µl) (Roche)

RNA polymerase Sp6 (20 u/µl) (Roche)

RNA polymerase T7 (20 u/µl) (Roche)

RNAse-out (40 u/µl) (Roche)

Sheep serum (Sigma)

Sodium acetate

Superscript III (200 u/µl) (Invitrogen)

T4 DNA ligase (4 u/µl) (Invitrogen)

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

Triethylamine (TEA) (Fisher)

Triton X-100 (VWR)

Trizol (Invitrogen)

Tween 20 (Sigma)

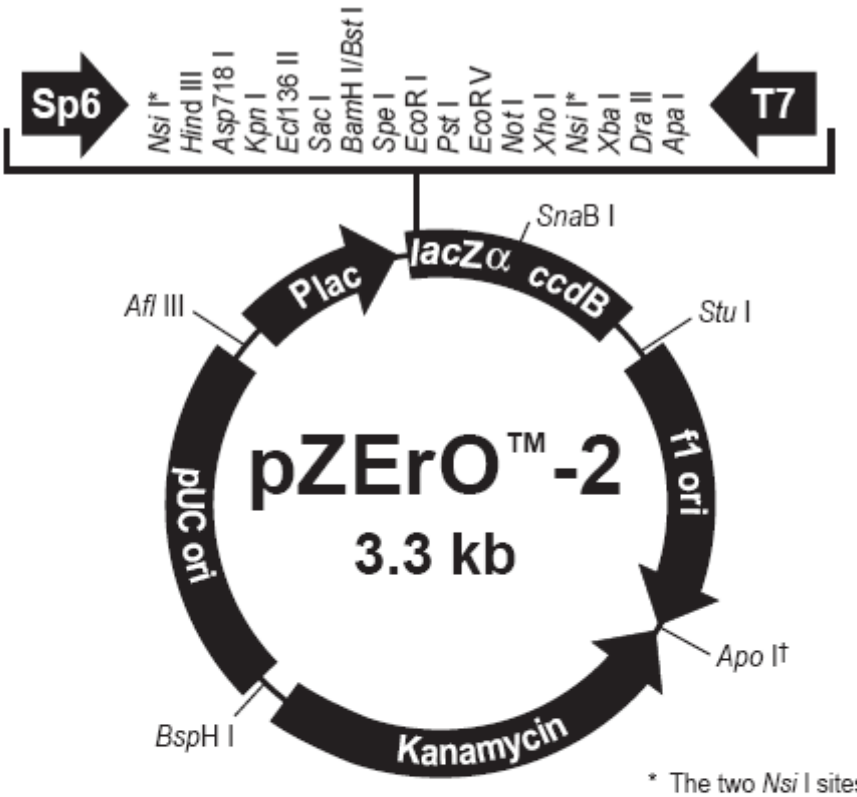
III Equipment

Name	Model	Manufacturer
Binocular	Wild M3B	Wild
Cameras	Axiocam MRm	Zeiss
	3CCD Mikroskopkamera	Sony
Centrifuges	Centrifuge 5410	Eppendorf
	Centrifuge 5417R	Eppendorf
	Sigma 3K20	B. Braun
	IEC MICROCL 17/17R	Thermo electro corporation
Confocal laser scanning microscope	TCS SPII	Leica
	LSM510	Zeiss
Elektroporator	Easyject Prima	Peqlab Biotech GmbH
Fluorescent binocular	MzFLIII	Leica
Fluorescent microscope	BX 50 WI	Olympus
Gel camera	Quickstore	MS Laborsysteme
Gel electrophoreses chambers	HE 33	Hoefer
	HE 99x	
	EV243	Jencons
Heat block	TB1 Thermoblock	Biometra
	Thermomixer	Eppendorf
	QBD1	Grant
Horizontal puller	Modell P97	Sutter
Incubators	KB-115	WTB Binder
	Friocell 707	MMM Medcenter Einrichtungen
Light source for Binocular	Highlight 3100	Olympus
Magnetic stirrer	MR3001	Heidolph
Micromanipulator		Leitz
Microinjection unit	FemtoJet	Eppendorf

Name	Model	Manufacturer
PCR machine	TGradient	Biometra
	TC-512	Techne
ph-meter	CG840	Schott
	3510 pH Meter	Jenway
Photometer	Biophotometer	Eppendorf
Pipettes	Pipetman 10, 20, 100, 200, 1000	Gilson
Power supply for gel electrophoreses	Gene Power Supply GPS 200/400	Pharmacia
Scales	PM 4600 Delta Range	Mettler
	PLJ360-3M	Kern
Shaker	Shaker Dos 20S	neoLab
	REAX2	Heidolph
	VX7	Janke&Kunkel
Overhead shaker	Rotator SB3	Stuart
Transmission microscope	Axioplan	Zeiss
	Axioskop 2	Zeiss
UV table	Transilluminator N90 M	MS Laborgeräte
Vacuum centrifuge	Savant Speed Vac	Thermo Life Sciences
Vortexer	Vibrofix VF1	Janke&Kunkel
	Vortex Genie 2TM	Bender&Hobein AG
Waterbath	SUB Aqua 12	Kötterman Grant

Table 7-1: Equipment used in preparation of this thesis.

IV Cloning vector



Comments for pZErO™-2
3297 nucleotides

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

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

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

V Sequences

Netrin alignment across various species

The extent of the conserved domains is indicated above the alignment. These are an N-terminal signaling peptide (**SS**, signal sequence), two domains homologous to laminin, containing three EGF-repeats (**VI** and **V-1**, **2**, **3**) and a C-terminal region (**C**).

```
                          ←SS  VI →
               ....|....| ....|....| ....|....| ....|....| ....|....|
                        10         20         30         40         50
Dm Netrin-A    -MIRGILLLL LGTTRFSPIQ CISNDV--YF KMFSQQAPPE DPCYNKAHEP
Dm Netrin-B    -MVR------ ATGTRMGLLL PIILAL--AI GSSAAGISSN DPCYFEG-KP
Tc netrin      -----MCSAV NMLLLLVIVP VVICVSDNFL KMFEGQKPPV DPCYDED-RP
At netrin      ---------- ---------- ---------- ---------- ----------
Cs netrin      -------WQF RDYRYSSYAS SLVPSSDPLV TAAS----VL EFCR------
Pd netrin      MRPQSCRRQA LGLLMVLAIA GSCRGASSFL NMFVQQ-NPP DPCYEEGEKP
Mm netrin-1    -MMRAVWEAL AALAAVACLV GAVRGG-PGL SMFAGQAAQP DPCSDENGHP
Gg netrin-1    MPRRGAEGPL ALLLAAAWLA QPLRGGYPGL NMFAVQTAQP DPCYDEHGLP
Dr netrin-1    --MIRILVTC VSMVSITSMV SGARGG-YGM SMFAAQSSPP DPCYDENGNP
Xl netrin-1    ---------- ---------- ---------- -------AQP DPCYDENGHP
Hs netrin-1    -MMRAVWEAL AALAAVACLV GAVRGG-PGL SMFAGQAAQP DPCSDENGHP

               ....|....| ....|....| ....|....| ....|....| ....|....|
                        60         70         80         90        100
Dm Netrin-A    RACIPDFVNA AYDAPVVASS TCGSSGAQRY CEYQD---HE RS--CHTCDM
Dm Netrin-B    RKCLPSFVNA AYGNPVQASS VCGAQQPERY CELLR---DG NAGECRSCEQ
Tc netrin      RRCIPDFVNA AFGIPVKASS TCGLHSPVQF CEVSEPEG-- -NSQCYICDD
Pd netrin      RACIPGFENC AFGKEVKASS TCGAP-PSRY CQTVANKDGK MARNCFICDA
At netrin      ---------- ---------- ---------- --SNDDKGRD RSQFARYVTP
Cs netrin      CRCIPDFVNA AFGKEVKASS ECGTPANRYC TTSNDDKG-E IVRNCQICDA
Mm netrin-1    RRCIPDFVNA AFGKDVRVSS TCGRP-PARY CVVSE-RGEE RLRSCHLCNS
Gg netrin-1    RRCIPDFVNS AFGKEVKVSS TCGKP-PSRY CVVTE-KGEE QVRSCHLCNA
Dr netrin-1    RRCIPDFVNS AFGKDVRVSS TCGSP-PSRC CRVTE-KGEE RSRDCNICDA
Xl netrin-1    RRCIPDFVNS AFGKEVKVSS TCGKP-PSRY CVVTE-KGED RFRNCHICNM
Hs netrin-1    RRCIPDFVNA AFGKDVRVSS TCGRP-PARY CVVSE-RGEE RLRSCHLCNA

               ....|....| ....|....| ....|....| ....|....| ....|....|
                       110        120        130        140        150
Dm Netrin-A    TDPLRSFPAR SLTDLNNSNN VTCWRS---- ---EPVT--- ---GSGDNVT
Dm Netrin-B    ----QRYGPA ALTDLNNPSN VTCWRSGAVN VPHDPDS--- ---APPDNVT
Tc netrin      KLPKNRFPAS HLTDLNNPNN VTCWRS---- ---DPLLPVT SINAAPDNVT
Pd netrin      NHPRRQHPVG FLTDLNNPNN LTCWMS---- ---EPFV--- ---QFPQNVT
At netrin      AIPKRAHPTS YLTDLNNPNN VTCWMS---- ---EPFS--- ---QSLHNVS
Cs netrin      SNPKRAHPTS YLTDLNNPNN VTCWMS---- ---EPFS--- ---QSLHNVS
Mm netrin-1    SDPKKAHPPA FLTDLNNPHN LTCWQS---- ---ENYL--- ---QFPHNVT
Gg netrin-1    SDPKRAHPPS FLTDLNNPHN LTCWQS---- ---DSYV--- ---QYPHNVT
Dr netrin-1    TDPKKTHPPA YLTDLNNPHN LTCWQS---- ---ENYV--- ---QYPQNVT
Xl netrin-1    SDAKRAHPPS FLTDLNNPHN LTCWQS---- ---ENYI--- ---QYPQNVT
Hs netrin-1    SDPKKAHPPA FLTDLNNPHN LTCWQS---- ---ENYL--- ---QFPHNVT
```

```
                 ....|....| ....|....| ....|....| ....|....| ....|....|
                        160        170        180        190        200
Dm Netrin-A      LTLSLGKKFE LTYVILQLCP HAPRPDSMVI YKSTDHGLSW QPFQFFSSQC
Dm Netrin-B      LTLSLGKKYE LTYISLSFCP RSPRPDSLAI FKSSDFGQTW QPFQFYSSQC
Tc netrin        LVLSLGKKYE LTYVSLQFCP NTPKPDSIAI YKSMDYGKTW QAFQFYSTQC
Pd netrin        LTLSLGKKYE LTYVSLQFCS --ARPDSMAI YKSVDYGKTW IPFQFYSSQC
At netrin        LTLSLGKKYE LTYISLQFCN --QKPDSLAL YKSMDYGKTR HPFQFYSSQC
Cs netrin        LTLSLGKKYE LTYISLQFCN --QKPDSLAL YKSMDYGKTW HPFQFYSSQC
Mm netrin-1      LTLSLGKKFE VTYVSLQFCS --PRPESMAI YKSMDYGRTW VPFQFYSTQC
Gg netrin-1      LTLSLGKKFE VTYVSLQFCS --PRPESMAI YKSMDYGKTW VPFQFYSTQC
Dr netrin-1      LTLSLGKKFE VTYVSLQFCS --PRPESMAI FKSMDYGKTW VPFQFYSTQC
Xl netrin-1      LTLSLGKKFE VTYVSLQFCS --PRPESMAI FKSMDYGKSW VPFQFYSTQC
Hs netrin-1      LTLSLGKKFE VTYVSLQFCS --PRPESMAI YKSMDYGRTW VPFQFYSTQC

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                        210        220        230        240        250
Dm Netrin-A      RRLFGRPARQ STGRHNEHEA RCS---DVTR PLVS------ RIAFSTLEGR
Dm Netrin-B      QKFYGRPDRA KISKFNEQEA RCINSQHDTG GAAQ------ RFAFNTLEGR
Tc netrin        RRVYGRPNKA TITKVNEQEA RCTDAHRYTG GDNQGFGQSS RIAFSTLEGR
Pd netrin        KKMYGKSPRA VITRANEQEA LCTDAYSNID PLSGA----- RVAFSTLEGR
At netrin        RKVYGRQNRA AITKANEQEA LCTDAQSNVE PITG-----A GIAFSTLEGR
Cs netrin        RKMYGRQNRA AITKANEQEA LCTDAQSNVE PITG-----A RIAFSTLEGR
Mm netrin-1      RKMYNRPHRA PITKQNEQEA VCTDSHTDMR PLSGG----- LIAFSTLDGR
Gg netrin-1      RKMYNKPSRA AITKQNEQEA ICTDSHTDVR PLSGG----- LIAFSTLDGR
Dr netrin-1      KKMYNKPSKA AITKQNEQEA ICTDSHTDMQ PLTGG----- LIAFSTLDGR
Xl netrin-1      RKMYNKPNKA IITKQNEQEA ICTDSHTDMH PLSGG----- LIAFSTLDGR
Hs netrin-1      RKMYNRPHRA PITKQNEQEA VCTDSHTDMR PLSGG----- LIAFSTLDGR

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                        260        270        280        290        300
Dm Netrin-A      PSSRDLDSSP VLQDWVTATD IRVVFHRLQR P--------- ----------
Dm Netrin-B      PSANDLDSSL VLQDWVTATD IRVVFHRLEL PPQLLKVKNA NAFSDEMGGS
Tc netrin        PSALDFDNSP VLQDWITATD IKIIFNRLYM PLE------- ----------
Pd netrin        PSAYDFDNSP VLQDWVTATD IQVVFNRLNT Y--------- ----------
At netrin        PSAYDFDNSP VLQDWVTATD IKVMFNRLVG ---------- ----------
Cs netrin        PSAYDFDNSP VLQDWVTATD IKVMFNRLVG ---------- ----------
Mm netrin-1      PSAHDFDNSP VLQDWVTATD IRVAFSRLHT F--------- ----------
Gg netrin-1      PTAHDFDNSP VLQDWVTATD IKVTFSRLHT F--------- ----------
Dr netrin-1      PSAHDFDNSP VLQDWVTATD IKVTFNRLHT F--------- ----------
Xl netrin-1      PSAHDFDNSP VLQDWVTATD IKVAFSRLHT F--------- ----------
Hs netrin-1      PSAHDFDNSP VLQDWVTATD IRVAFSRLHT F--------- ----------

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                        310        320        330        340        350
Dm Netrin-A      -----DPQAL LSLEAG---- -------GAT DLASGKYSVP ----------
Dm Netrin-B      REEDEDDDAD LELDGEQDEY DYNLQDNDSA DAGYDEYEEP KKHLELDDDH
Tc netrin        ---------- ---------- --DASSENFD TLVDELKNKQ TDDDLNFTKE
Pd netrin        ---------- ---------- ---------- ---------- ----------
At netrin        ---------- ---------- ---------- ---------- ----------
Cs netrin        ---------- ---------- ---------- ---------- ----------
Mm netrin-1      ---------- ---------- ---------- ---------- ----------
Gg netrin-1      ---------- ---------- ---------- ---------- ----------
Dr netrin-1      ---------- ---------- ---------- ---------- ----------
Xl netrin-1      ---------- ---------- ---------- ---------- ----------
Hs netrin-1      ---------- ---------- ---------- ---------- ----------
```

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                ....|....| ....|....| ....|....| ....|....| ....|....|
                       360        370        380        390        400
Dm Netrin-A     --LANGPAGN NIEANLG--- ---------- ---------- ----------
Dm Netrin-B     LHLDYASDGE SVVKRQGKHK GSAYEKHYQS KLAATTPPQQ PPKVTPPGKV
Tc netrin       PVVQLVNEMQ PLDTNDPK-- ---------- ---------- ----------
Pd netrin       ---GDE---D DQQARES--- ---------- ---------- ----------
At netrin       WMPEMANDNE SLSQRD---- ---------- ---------- ----------
Cs netrin       WMPEMANDNE SLSQRD---- ---------- ---------- ----------
Mm netrin-1     ---GDENEDD SELARDS--- ---------- ---------- ----------
Gg netrin-1     ---GDENEDD SELARDS--- ---------- ---------- ----------
Dr netrin-1     ---GDENEDD SELARDS--- ---------- ---------- ----------
Xl netrin-1     ---GDENEDD SELARDS--- ---------- ---------- ----------
Hs netrin-1     ---GDENEDD SELARDS--- ---------- ---------- ----------

                                                ← VIV-1 →
                ....|....| ....|....| ....|....| ....|....| ....|....|
                       410        420        430VIV-1   440        450
Dm Netrin-A     ---GDVATSG SG-------- LHYAISDFSV GGRCKCNGHA SKCSTDASG-
Dm Netrin-B     TPPSTAAPSA AASAVTLPIS QHYAVSDFAV GGRCKCNGHA SECVATVSSG
Tc netrin       TPTSVELTSN GVAGGYNQIT HQYAVADFAV GGRCKCNGHA SRCITGRDG-
Pd netrin       ---------- ---------- YYYALSDFAV GGRCKCNGHA SRCVADDTG-
At netrin       ---------- ---------T YFYAVSDLAV GGRCKCNGH- ----------
Cs netrin       ---------- ---------T YFYAVSDLAV GGRCKCNGHA SRCTHNREG-
Mm netrin-1     ---------- ---------- YYYAVSDLQV GGRCKCNGHA ARCVRDRDD-
Gg netrin-1     ---------- ---------- YFYAVSDLQV GGRCKCNGHA SRCVRDRDD-
Dr netrin-1     ---------- ---------- YFYAVSDLQV GGRCKCNGHA SKCVKDREG-
Xl netrin-1     ---------- ---------- YFYAVSDLQV GGRCKCNGHA SRCVKDRDD-
Hs netrin-1     ---------- ---------- YFYAVSDLQV GGRCKCNGHA ARCVRDRTD-

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       460        470        480        490        500
Dm Netrin-A     ---------- ---------- ---------- ------QLNC ECSHNTAGRD
Dm Netrin-B     SGTALSDQDD GQDEDTPSAP SLANHFGRST QMSAKLTMTC ACKHNTAGPE
Tc netrin       ---------- ---------- ---------- ------QLAC ECKHNTAGKD
Pd netrin       ---------- ---------- ---------- ------KMTC DCKHNTAGVD
At netrin       ---------- ---------- ---------- ---------- ----------
Cs netrin       ---------- ---------- ---------- ------QLVC DCKHNTAGRD
Mm netrin-1     ---------- ---------- ---------- ------SLVC DCRHNTAGPE
Gg netrin-1     ---------- ---------- ---------- ------NLVC DCKHNTAGPE
Dr netrin-1     ---------- ---------- ---------- ------NLVC ECKHNTAGPE
Xl netrin-1     ---------- ---------- ---------- ------NLVC DCKHNTAGPE
Hs netrin-1     ---------- ---------- ---------- ------SLVC DCRHNTAGPE

                                      ← V-1 V-2 →
                ....|....| ....|....| ....|....| ....|....| ....|....|
                       510        520        530        540        550
Dm Netrin-A     CERCKPFHFD RPWARATAKE ANECKECN-- ---------- ----------
Dm Netrin-B     CERCKPFYFD RPWGRATDND ANECKMCQ-- ---------- ----------
Tc netrin       CERCKPFHFD RPWGRATARD ANECKVFENS KAEERMYVLT QNILWNLRKR
Pd netrin       CERCKSFHYD RPWARASAKE ANECVACK-- ---------- ----------
At netrin       ---------- ---------- ---------- ---------- ----------
Cs netrin       CEKCKPFHLT DRGESYSSRS PMCCMQLQLP RPPL-SFTWS FTTVW-----
Mm netrin-1     CDRCKPFHYD RPWQRATARE ANECVACN-- ---------- ----------
Gg netrin-1     CDRCKPFHYD RPWQRATARE ANECVACN-- ---------- ----------
Dr netrin-1     CDRCKPFHYD RPWQRATARE ANECVACH-- ---------- ----------
Xl netrin-1     CDRCKPFHYD RPWQRATARE ANECVACN-- ---------- ----------
Hs netrin-1     CDRCKPFHYD RPWQRATARE ANECVACN-- ---------- ----------
```

```
                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         560        570        580        590        600
Dm Netrin-A      ---------- ---------- ---------- ---------C NKHARQCRFN
Dm Netrin-B      ---------- ---------- ---------- ---------C NGHARRCRFN
Tc netrin        LKNNEQKMVP NADPFLRLNS KTITRFTSFV TSINSETCEC NQHARRCRFN
Pd netrin        ---------- ---------- ---------- ---------C NLHARQCQFN
At netrin        ---------- ---------- ---------- ---------- ----------
Cs netrin        ---------- -PDQWRRLP- ---------- ---------- ----------
Mm netrin-1      ---------- ---------- ---------- ---------C NLHARRCRFN
Gg netrin-1      ---------- ---------- ---------- ---------C NLHARRCRFN
Dr netrin-1      ---------- ---------- ---------- ---------C NLHARRCRFN
Xl netrin-1      ---------- ---------- ---------- ---------C NLHARRCRFN
Hs netrin-1      ---------- ---------- ---------- ---------C NLHARRCRFN

                                                          ←V-2
                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         610        620        630        640        650
Dm Netrin-A      MEIFRLSQGV SGGVCQNCRH STTGRNCHQC KEGFYRDATK PLTHRKVCKA
Dm Netrin-B      LELYKLSGRV SGGVCYNCQH DTTGRYCHYC REGYYRDATK PPNHRKVCKR
Tc netrin        MELYKLSGRV SGGVCLKCRH YTAGRHCHYC REGFYRDPTK QITHRKACKP
Pd netrin        MELYKLSGRK SGGVCLNCKH NTAGRNCHYC KEGYYRDQAK PITHRKACKA
At netrin        ---------- ---------- ---------- ---------- ----------
Cs netrin        ---------- ---------- ---------- ---------- ----------
Mm netrin-1      MELYKLSGRK SGGVCLNCRH NTAGRHCHYC KEGFYRDMGK PITHRKACKA
Gg netrin-1      MELYKLSGRK SGGVCLNCRH NTAGRHCHYC KEGFYRDLSK PISHRKACKE
Dr netrin-1      MELYKLSGRR SGGVCLNCRH NTAGRHCHYC KEGYYRDMSK AISHRRACKA
Xl netrin-1      MELFKLSGRR SGGVCLNCRH NTAGRHCHYC KEGYYRDMTK AITHRKACKA
Hs netrin-1      MELYKLSGRK SGGVCLNCRH NTAGRHCHYC KEGYYRDMGK PITHRKACKA

                 V-3 →                                              ← V-3
                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         660        670        680        690        700
Dm Netrin-A      CDCHPIGSSG KICNSTSGQC PCKDGVTGLT CNRCARGYQQ SRSHIAPCIK
Dm Netrin-B      CDCHPVGSTG KTCNHLSGQC PCKEGVTGLT CNRCARGYQQ TRSHVAPCIK
Tc netrin        CDCHPIGSSG RTCNQATGQC PCKDGVVGTT CNRCAKGYQQ SRSHIAPCIK
Pd netrin        CDCHPVGALG KTCNQTTGQC PCKDGVTGVT CNRCAKGYQQ SRSPIAPCIK
At netrin        ---------- ---------- ---------- ---------- ----------
Cs netrin        -ECRPHGWT- ---TPATTQK ATRD------ --------KP SHSHQR----
Mm netrin-1      CDCHPVGAAG KTCNQTTGQC PCKDGVTGIT CNRCAKGYQQ SRSPIAPCIK
Gg netrin-1      CDCHPVGAAG QTCNQTTGQC PCKDGVTGIT CNRCAKGYQQ SRSPIAPCIK
Dr netrin-1      CDCHPVGAAG KTCNQTTGQC PCKDGVTGIT CNRCAKGYQQ SRSPIAPCIK
Xl netrin-1      CDCHPVGAAG KTCNQTTGQC PCKDGVTGIT CNRCAKGYQQ SRSPIAPCIK
Hs netrin-1      CDCHPVGAAG KTCNQTTGQC PCKDGVTGIT CNRCAKGYQQ SRSPIAPCIK

                 C →
                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         710        720        730        740        750
Dm Netrin-A      QPPRMINMLD TQNTAPEPDE PESSPGSGGD RNGAAGMAAQ SQYYRTEGGR
Dm Netrin-B      VP-TNANMIQ AESAGGG--- --GGGGTGDY KDGGGSQVEE MKKY------
Tc netrin        IPVVQIMATE EEDNDYGDED PDQPAAG--- ---------- ---------V
Pd netrin        IPKPTQPEWH RTNNAGP--- --TPRGE--- ---------- ----------
At netrin        ---------- ---------- ---------- ---------- ----------
Cs netrin        LQAV------ ---------- ---------- ---------- ----------
Mm netrin-1      IP-VAPPTTA ASSVEEP--- --EDCDS--- ---------- ----------
Gg netrin-1      IP-AAPPPTA ASSTEEP--- --ADCDS--- ---------- ----------
Dr netrin-1      IP-VAAPTSS YSSPDGP--- --TDCDS--- ---------- ----------
Xl netrin-1      IP-VVPPTTA ASSTEEP--- --ADCDI--- ---------- ----------
Hs netrin-1      IP-VAPPTTA ASSVEEP--- --EDCDS--- ---------- ----------
```

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                       760        770        780        790        800
Dm Netrin-A     ECGKCRVSTK RLNLNKFCKR DYAIMAKVIG RDTSSEAVSR EVQRRAMDPD
Dm Netrin-B     -CGKCKASPK KLNLNKFCME DYAILAKVIG HDRASQDIS- ----------
Tc netrin       QCGKCKSSTK RLNLNKYCKR DYAIMARVLS RMEGNEDQHT ----------
Pd netrin       -CGKCKTSRR KINIKKYCRR DYAIQVEILQ RETVG----- ----------
At netrin       ---------- ---------- ---------- ---------- ----------
Cs netrin       ---------- ---------- ---------- ---------- ----------
Mm netrin-1     -YCKASKGKL KMNMKKYCRK DYAVQIHILK ADKAG----- ----------
Gg netrin-1     -YCKASKGKL KINMKKYCKK DYAVQIHILK AEKNA----- ----------
Dr netrin-1     -HCKAPKGKM KVTMKKYCKK DFAVQVHVLK GDKAG----- ----------
Xl netrin-1     -YCKASKGKL KINMKKYCKK DYAVQIHILK AEKAG----- ----------
Hs netrin-1     -YCKASKGKL KINMKKYCKK DYAVQIHILK ADKAG----- ----------


                ....|....| ....|....| ....|....| ....|....| ....|....|
                       810        820        830        840        850
Dm Netrin-A     VADYEMDQVQ PGSARSPITG VYEFQAADYP NPNPNPRGSE MERFDLQIQA
Dm Netrin-B     ---------- --------TE KFSIE----- ------RQNE IYKYEINIQT
Tc netrin       ---------- ---------- ---------- --------KG WTRFMVNIEF
Pd netrin       ---------- ---------- ---------- ---------D WVKFPVNIIT
At netrin       ---------- ---------- ---------- ---------- ----------
Cs netrin       ---------- ---------- ---------- ---------- ----------
Mm netrin-1     ---------- ---------- ---------- ---------D WWKFTVNIIS
Gg netrin-1     ---------- ---------- ---------- ---------D WWKFTVNIIS
Dr netrin-1     ---------- ---------- ---------- ---------E WWKFTINIIS
Xl netrin-1     ---------- ---------- ---------- ---------D WWKFTVNVIS
Hs netrin-1     ---------- ---------- ---------- ---------D WWKFTVNIIS


                ....|....| ....|....| ....|....| ....|....| ....|....|
                       860        870        880        890        900
Dm Netrin-A     VFKRSRPGES SGAGNVYGMP NTTLKRGPMT WIIPTKDLEC RCPRIRVNRS
Dm Netrin-B     IFKRNP---- -----MSGTT SSLLGRGNMM LLVPRKSIEC QCPKIKLNKS
Tc netrin       IYKKSR---- ---------- DSQIRKGTMP MVVPTADLAC KCPKLKPSKS
Pd netrin       VYKRGQ---- ---------- -ERVARGENA LWLPLSDLMC KCPKVRVNKR
At netrin       ---------- ---------- ---------- ---------- ----------
Cs netrin       ---------- ---------- ---------- ---------- ----------
Mm netrin-1     VYKQGT---- ---------- -SRIRRGDQS LWIRSRDIAC KCPKIKPLKK
Gg netrin-1     VYKQGS---- ---------- -NRLRRGDQT LWVHAKDIAC KCPKVKPMKK
Dr netrin-1     VYKQGG---- ---------- -HRIRRGDQL LWVRAKDVAC KCPKIKPGRK
Xl netrin-1     VYKQGT---- ---------- -NRIRRGDQN LWIRSKDIAC KCPKLKPMKK
Hs netrin-1     VYKQGT---- ---------- -SRIRRGDQS LWIRSRDIAC KCPKIKPLKK

                                                        ← C
                ....|....| ....|....| ....|....| ....|....| ....|....|
                       910        920        930        940        950
Dm Netrin-A     YLILGRDS-- ---EAPPGYL GIGPHSIVIE WKEDWYRRMK RFQRRARTCA
Dm Netrin-B     YLILGRDS-- ---EAAPGYL AIGPSSVVLE WKDEWSLRMK RFQRRARKCS
Tc netrin       YLFLGREKDD SPGGVLTGSL GVTERSIVIE WRDEWDQRMR RFRRRARKCK
Pd netrin       YLVL------ ---------- ---------- ---------- ----------
At netrin       ---------- ---------- ---------- ---------- ----------
Cs netrin       ---------- ---------- ---------- ---------- ----------
Mm netrin-1     YLLLGNAE-- --DSPDQSGI VADKSSLVIQ WRDTWARRLR KFQQREKKGK
Gg netrin-1     YLLLGSTE-- --DSPDQSGI IADKSSLVIQ WRDTWARRLR KFQQREKKGK
Dr netrin-1     YLLMGSDEEE --DSRGQSGV VADRGSLLIP WKDLWARRLR KFQQRDKRGK
Xl netrin-1     YLLLGNDE-- --DSPDQNGV VADKTSLVIQ WRDTWARRLR KFQQREKKGK
Hs netrin-1     YLLLGNAE-- --DSPDQSGI VADKSSLVIQ WRDTWARRLR KFQQREKKGK
```

Figure 7-1: Amino acid alignment of the conserved regions of *netrin* in various species.
Legend see next page.

Legend to Figure 7-1: Amino acids that are identical in more than 6 of the listed species are highlighted in grey. The evolutionary conserved are shown above the sequence. ***At***; *Achaearanea tepidariorum*, ***Cs***; *Cupiennius salei*, ***Dm***; *Drosophila melanogaster*, ***Dr***; *Danio rerio*, ***Gg***; *Gallus gallus*, ***Hs***; *Homo sapiens*, ***Mm***; *Mus musculus*, ***Tc***; *Tribolium castaneum*, ***Pd***; *Platynereis dumerilii*, ***Xl***; *Xenopus laevis*.

Netrin alignment in spiders

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                ....|....| ....|....| ....|....| ....|....| ....|....|
                        110        120        130        140        150
At netrin       ATGACAAGGG GAGAGATCGT TCGCAATTTG CCAGATATGT GACGCCAGCA
Cs netrin       ACGACAAGGG -AGAGATCGT TCGCAATT-G CCAGATATGT GACGCCAGCA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        160        170        180        190        200
At netrin       ATCCCGAAGC GCGCTCATCC CACGAGCTAC TTGACAGATC TCAACAACCC
Cs netrin       ATCC-GAAGC GCGCTCATCC CACGAGCTAC TTGACAGATC TCAACAACCC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        210        220        230        240        250
At netrin       GAACAATGTC ACATGCTGG- ATGTCCGAAC CTTTCTCGC- AGTCTTTGCA
Cs netrin       GAACAATGTC ACATGCTGG- ATGTCCGAAC CTTTCTCGC- AGTCTTTGCA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        260        270        280        290        300
At netrin       -CAACGTGTC T-CTGACACT GTCTCTGGGC AAGAAGTACG AGCTGACGTA
Cs netrin       -CAACGTGTC T-CTGACACT GTCTCTGGGC AAGAAGTACG AGCTGACGTA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        310        320        330        340        350
At netrin       CATAAGCC-T GCAGTTCTGC AATCAGAAGC CAGACTCTCT GGCTCTTTAC
Cs netrin       CATAAGCC-T GCAGTTCTGC AATCAGAAGC CAGACTCTCT GGCTCTTTAC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        360        370        380        390        400
At netrin       AAGAGCATGG ACTACGGAAA GACACGGCAT CCATTCCAGT TCTATTCGAG
Cs netrin       AAGAGCATGG ACTACGGAAA GACATGGCAT CCATTCCAGT TCTATTCGAG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        410        420        430        440        450
At netrin       TCAGTGCCGG AAGGTGTACG GTCGCCAAAA CAGGGCCGCC ATTACAAAGG
Cs netrin       TCAGTGCCGG AAGATGTACG GTCGCCAAAA CAGGGCCGCC ATTACAAAGG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        460        470        480        490        500
At netrin       CTAACGAGCA GGAAGCGCTC TGCACCGATG CCCAGTCAAA TGTTGAGCCC
Cs netrin       CTAACGAGCA GGAAGCGCTC TGCACCGATG CCCAGTCAAA TGTTGAGCCC
```

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                       510        520        530        540        550
At netrin       ATAACAGGTG CCGGGATTGC GTTTAGCACT CTGGAAGGCC GGCCGTCTGC
Cs netrin       ATAACAGGTG CCAGGATTGC GTTTAGCACT CTGGAAGGCC GGCCGTCTGC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       560        570        580        590        600
At netrin       CTACGACTTC GACAACAGCC CAGTGCTGCA AGACTGGGTG ACGGCAACCG
Cs netrin       CTACGACTTC GACAACAGCC CAGTGCTGCA AGACTGGGTG ACGGCAACCG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       610        620        630        640        650
At netrin       ACATTAAAGT CATGTTCAAC AGGCTGGTGG GGTGGATGCC AGAGATGGCA
Cs netrin       ACATTAAAGT CATGTTCAAC AGGCTGGTGG GGTGGATGCC AGAGATGGCA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       660        670        680        690        700
At netrin       AACGATAATG AATCCCTAAG CCAGAGAGAC ACGTACTTCT ACGCTGTCTC
Cs netrin       AACGATAATG AATCCCTAAG CCAGAGAGAC ACGTACTTCT ACGCTGTCTC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       710        720        730        740        750
At netrin       TGACCTCGCT GTTGGAGGCA GATGCAAGTG TAACGGTCAC G---------
Cs netrin       TGACCTCGCT GTTGGAGGCA GATGCAAATG CAATGGACAT GCCTCGAGAT
```

Figure 7-2: Nucleotide sequence alignment of *netrin* in the spiders *Achaearanea tepidariorum* and *Cupiennius salei*.

The alignment for *netrin* isolated in *Cupiennius* and *Achaearanea* reveals that in both spider species the nucleotide sequence is almost perfectly identical. Identical nucleotides are highlighted in grey.

Alignment of *single minded*

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                    ....|....| ....|....| ....|....| ....|....| ....|....|
                             10         20         30         40         50
Atsim AB236150      CAGTTTTGGC TTACTCGGGA CCGCAGCTAG AAATCTTTAA ACCGATCAGT
Atsim clone8        ---------- ---------- ---------- ---------- ----------

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                             60         70         80         90        100
Atsim AB236150      CATTTAAAAA GAACAATAAT ATTCTAGAAT AAATAAGAAT TAACAGGAGT
Atsim clone8        ---------- ---------- ---------- ---------- ----------

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            110        120        130        140        150
Atsim AB236150      AATAATCAAC AAATATTAAG AAGAATGTAA GAGGTATGGG ATAGTATCAA
Atsim clone8        ---------- ---------- ---------- ---------- ----------
```

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                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         160        170        180        190        200
Atsim AB236150   AAAGAAATTA ACTCGATTTA ATTTTTTAAA TTTTTCAAAG ATGAATTGAA
Atsim clone8     ---------- ---------- ---------- ---------- ----------

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         210        220        230        240        250
Atsim AB236150   ACTGTGTCGA ATATTTTTCT CAAGTATGCT TGGTCAGTAA TGGGATGACT
Atsim clone8     ---------- ---------- ---------- ---------- ----------

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         260        270        280        290        300
Atsim AB236150   TATTAAAACC AATTGTAAAG TGAATTTGAG AAACTGATAA TATTCTTTGC
Atsim clone8     ---------- ---------- ---------- ---------- ----------

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         310        320        330        340        350
Atsim AB236150   AAAAAGAGAG TTCCTTATAT TGCGGTTGCC ATTCTCAATT AAGGGCAATG
Atsim clone8     ---------- ---------- ---------- ---------- ----------

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         360        370        380        390        400
Atsim AB236150   TAAAGTAACG TGTCAAAATT TCGTCAAAAA GAACATCCAG GCTAGCGAGG
Atsim clone8     ---------- ---------- ---------- ---------- ----------

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         410        420        430        440        450
Atsim AB236150   AATAATCTTT CCTTGCTTTG CGAACAGAAT GAAGGAGAAA TCCAAAAATG
Atsim clone8     ---------- ---------- ---------- ---------- ----------

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         460        470        480        490        500
Atsim AB236150   CTGCTCGCTC TCGCCGAGAG AAAGAAAACG CTGAGTTTTT AGAACTGGCT
Atsim clone8     ---------- ---------- ---------- ---------- ----------

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         510        520        530        540        550
Atsim AB236150   AAACTCCTTC CACTTCCTCC CGCCATTACT AGCCAACTGG ACAAAGCATC
Atsim clone8     ---------- ---------- ---------- ---------- ----------

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         560        570        580        590        600
Atsim AB236150   CATCATCCGT CTCACAACAT CCTACCTAAG GATGAGGGAA GTCTTTCCTA
Atsim clone8     ---------- ---------- ---------- ---------- ----------

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         610        620        630        640        650
Atsim AB236150   AAGGTCTTGG GGATAGCTGG GGAGCAGCTC CTCCATTGCC AAACCCCTTA
Atsim clone8     ---------- ---------- ---------- ---------- ----------
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                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         660        670        680        690        700
Atsim AB236150   GAAAGTGCAA TAAAAGAACT TGGTTCTCAT CTCTTGCAGA CGCTGGATGG
Atsim clone8     ---------- ---------- ---------- ---------- ----------

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         710        720        730        740        750
Atsim AB236150   TTTTGTTTTT GTTGTTGCTC CAGATGGGAA AATAATGTAT ATTTCTGAAA
Atsim clone8     ---------- ---------- ---------- ---------- ----------

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         760        770        780        790        800
Atsim AB236150   CTGCTTCCGT CCATCTAGGA TTATCACAAG TAGAGTTGAC AGGCGACAGC
Atsim clone8     ---------- ---------- ---------- ---------- ----------

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         810        820        830        840        850
Atsim AB236150   ATTTATGAAT ACATCGATCC AACTGACCAT GACGAGATGG CTGCTGTCTT
Atsim clone8     ---------- ---------- ---------- ---------- ----------

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         860        870        880        890        900
Atsim AB236150   GTCCCTTCAG ACACCACCTG TTCATCCTCA AATTCCTGCA CCACAGGGTG
Atsim clone8     ---------- ---------- ---------- ---------- ----------

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         910        920        930        940        950
Atsim AB236150   AATTTGAGCT GGAGAGATTG TTCTTTGTGA GGATGAAATG CGTTCTGGCC
Atsim clone8     ---------- ---------- -------TTT GGGTGACCTA TAGAATACTC

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         960        970        980        990       1000
Atsim AB236150   AAGCGAAACG CTG-GTTTGA CATCGGGTGG ATACAAGGTC ATTCACT-GT
Atsim clone8     AAGCTATGCA TCAAGCTTGG TACCGAG--- ---CTCGG-- ATCCACTAGT

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                        1010       1020       1030       1040       1050
Atsim AB236150   AGTGGGTATT TGAAAGTGCA ACGGTACAAC GTGGAAGCGC CCCCATATGA
Atsim clone8     AACGGCCGCC ----AGTGTG CTGGAATTCT GCAGATGCGC CCCCATATGA

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                        1060       1070       1080       1090       1100
Atsim AB236150   CAGTTGCTAT CAAAACTTGG GTTTGGTGGC AGTTGGTCAT TCCTTACCAC
Atsim clone8     CAGTTGCTAT CAAAACTTGG GTTTGGTGGC AGTTGGTCAT TCCTTACCAC

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                        1110       1120       1130       1140       1150
Atsim AB236150   CCAGCGCCAT CACCGAAATA AAGATGTACT CCAACATGTT CATGTTCCGA
Atsim clone8     CCAGCGCCAT CACCGAAATA AAGATGTACT CCAACATGTT CATGTTCCGA
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                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         1160       1170       1180       1190       1200
Atsim AB236150   GCCAACATGG ATCTCAGATT GATATTTCTA GATGCCAGAG TCACAAATTT
Atsim clone8     GCCAACATGG ATCTCAGATT GATATTTCTA GATGCCAGAG TCACAAATTT

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         1210       1220       1230       1240       1250
Atsim AB236150   AACCGGTTAC CAGCCTCAGG ATCTCATAGA AAAAACCTTG TATCACTACA
Atsim clone8     AACCGGTTAC CAGCCTCAGG ATCTCATAGA AAAAACCTTG TATCACTACA

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         1260       1270       1280       1290       1300
Atsim AB236150   TTCACGCCTC AGATTGTGTA CAAATGCGAT ATTCCCACGA AACCTTACTT
Atsim clone8     TTCATGCCTC AGATTGTGTA CAAATGCGAT ACTCCCACGA AACCCTACTT

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         1310       1320       1330       1340       1350
Atsim AB236150   CATAAAGGGC AAGTAACAAC CAAATACTAT CGATTCCTAA CCAAAGATGG
Atsim clone8     CATAAAGGGC AAGTAACAAC TAAATACTAC CGATTCCTAA CCAAAGATGG

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         1360       1370       1380       1390       1400
Atsim AB236150   TGGTTGGATA TGGATGCAGA GCTATGCCAC CGTTGTCCAT AATACGAGAT
Atsim clone8     TGGTTGGATA TGGATGCAGA GCTATGTCAC CGTTGTCCAT AATACGAGAT

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         1410       1420       1430       1440       1450
Atsim AB236150   CTTCAAGACC TCACTGTATA GTAAGCGTGA ATTATGTTCT CGGCAAACAA
Atsim clone8     CTTCAAGACC TCACTGTATA GTAAGCGTGA ATTATGTTCT CAGCAAACAA

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         1460       1470       1480       1490       1500
Atsim AB236150   GAAGGTGAAA GTTTGCAATT GGAAATGGAA CAAATTAGGA AGCCAGAACC
Atsim clone8     GAAGGTGAAA GTTTGCAATT GGAAATGGAA CAAATTAGGA AGCCAGAACC

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         1510       1520       1530       1540       1550
Atsim AB236150   TCTCTATCCA GCTTCCGTTA CCACTACTCA AAGCAGTCCT ACGAATGGCA
Atsim clone8     TCTCTATCCA GCTTCCGTTG CCACTACTCA AAGCAGTCCT ACGAATGGCA

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         1560       1570       1580       1590       1600
Atsim AB236150   CAACAAATGC AACACCTACG AGAGTGCGAC ATCAGAAAAA TAAAAATCGA
Atsim clone8     CAACAAATGC AACACCTACG AGAGTGCGAC ATCAGAAAAA TAAAAATCGA

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         1610       1620       1630       1640       1650
Atsim AB236150   AAATCGCCTT ACTTGTATGA AGAGCCTAAC ATTGGATGCT TGAGAGGTGA
Atsim clone8     AAATCGCCTT ACTTGTATGA AGAGCCTAAC ATTGGATGCT TGAGAGGTGA
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                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         1660       1670       1680       1690       1700
Atsim AB236150   ATCGGTAGAT CCTTGTTTGT ATGAAATGAA TTCCTTTCCG TTGCACCATG
Atsim clone8     ATCGGTAGAT CCTTGTTTGT ATGAAATGAA TTCCTTTCCG TTGCACCATG

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         1710       1720       1730       1740       1750
Atsim AB236150   CTGCATTTCC TCCAGTGACC CATGCCCCTT ATCCAGAGAT GAATACGCCA
Atsim clone8     CTGCATTTCC TCCAGTGACC CATGCCCCTT ATCCAGAGAT GAATACGCCA

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         1760       1770       1780       1790       1800
Atsim AB236150   AATCAACCCT CTCAGTATTC TCACTCTCCC CATGTAGCTA TGGGTCTTCT
Atsim clone8     AATCAACCCT CTCAGTATTC TCACTCTCCC CATGTAGCTA TGGGTCTTCT

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         1810       1820       1830       1840       1850
Atsim AB236150   CGATCCTGCC AATGGTGGGT TTCTTCCCCA CTATACACAC CAGAGACTAT
Atsim clone8     CGATCCTGCC AATGGTGGGT TTCTTCCCCA CTACACACAC CAGAGACTAT

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         1860       1870       1880       1890       1900
Atsim AB236150   CACCACTACC TCCTCAAGTG GAATCTACTT ACGAAAGAGA CGATCGATAT
Atsim clone8     CACCACTACC TCCTCAAGTG GAATCTACTT ACGAAAGAGA CGATCGATAT

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         1910       1920       1930       1940       1950
Atsim AB236150   TCAATGCACA ATTTTGCATC TTTTCCTCCT TGTGAGGCAT CTGTCATATC
Atsim clone8     TCAATGCACA ATTTTGCATC TTTTCCTCCT TGTGAGGCAT CTGTCATATC

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         1960       1970       1980       1990       2000
Atsim AB236150   GTCCTCTCCA GATATTGAGC CAGACGCTCT TCAACCTCTT CCCTGCGTGA
Atsim clone8     GTCCTCTCCA GATAT-GAGC -AGACGCTCT TCAACCTCTT CCCTGTGTGA

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         2010       2020       2030       2040       2050
Atsim AB236150   TTCCATCTCA ACAAGATTTG TGTAACGCCA GTGTGGGATA CGTCCCTGGT
Atsim clone8     TTCCATCTCA C--AGATTTG TGCAACGCCA GTGTGGGATA CGTCCCTGGT

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         2060       2070       2080       2090       2100
Atsim AB236150   GCCCCAGCAG AGCCCCATGA AATAATTGTG CCCCATGTAA TATACAATAC
Atsim clone8     GCCC-AGCAG AGCCC-ATGA A--TATTGTG CCC-ATGTAT TATACATAAC

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         2110       2120       2130       2140       2150
Atsim AB236150   AACTACGAAT AGCGTCGGAC CTTTCAGCAG CCCTTCGGAC TCCATGCTTT
Atsim clone8     A--------- ---------- ---------- ---------- ----------
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                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         2160       2170       2180       2190       2200
Atsim AB236150   CAGAATCAGA TCTAGAAGGC TTTCAAAATG GAAGGGCAGA TTCTCCTCGT
Atsim clone8     ---------- ---------- ---------- ---------- ----------

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         2210       2220       2230       2240       2250
Atsim AB236150   TCTACTTCAT CCACATCTTC ACCCTCACAT CACCATCAGC AGCATAACAA
Atsim clone8     ---------- ---------- ---------- ---------- ----------

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         2260       2270       2280       2290       2300
Atsim AB236150   CAACAACAGC TCTTCGAAAC CATCAACAAG TGCCTTAGGA CCTGTGCTTC
Atsim clone8     ---------- ---------- ---------- ---------- ----------

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         2310       2320       2330       2340       2350
Atsim AB236150   GCAGCAGTGG TAATAGTCAC AGCAATCATT ACAAATCCGT CATTACCCCT
Atsim clone8     ---------- ---------- ---------- ---------- ----------

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         2360       2370       2380       2390       2400
Atsim AB236150   ACCAATCTGC AGAGTCCCAC GGGACCCACT GACTTTCTCA ACTCCCGGTT
Atsim clone8     ---------- ---------- ---------- ---------- ----------

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         2410       2420       2430       2440       2450
Atsim AB236150   AACGAAACAG CACCAGTTCC AGCAACGAAA CTTTGCCAGT TGCTCACCGA
Atsim clone8     ---------- ---------- ---------- ---------- ----------

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         2460       2470       2480       2490       2500
Atsim AB236150   CTGATTCCTC TTACAACGAT TCCTGTTACT ATGAGGCGGC ACGACAGCAG
Atsim clone8     ---------- ---------- ---------- ---------- ----------

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         2510       2520       2530       2540       2550
Atsim AB236150   CAATCACACC TCGATTCCAG CATTAAGTAC GCGTTATCCA AATGTGACAG
Atsim clone8     ---------- ---------- ---------- ---------- ----------

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         2560       2570       2580       2590       2600
Atsim AB236150   TTACACCGAT AAAACGTATT GTGTGCCATC GAGCAACGGA ACGAGTGACT
Atsim clone8     ---------- ---------- ---------- ---------- ----------

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         2610       2620       2630       2640       2650
Atsim AB236150   ATAGTTGCAA TAATGCGCAT GACAGTGTCC AACCTCAGTA TACAAGCGTC
Atsim clone8     ---------- ---------- ---------- ---------- ----------
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                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         2660       2670       2680       2690       2700
Atsim AB236150   ATCGTTGACG CGCAGCAGTA CCAGATGGCG AACGGTTTTG TCCACTGATT
Atsim clone8     ---------- ---------- ---------- ---------- ----------


                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         2710       2720       2730       2740       2750
Atsim AB236150   TTGCATTTTC TAGTCGTCCG AGAAGTGGTC GAATTAATGA ACATATGTGC
Atsim clone8     ---------- ---------- ---------- ---------- ----------


                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         2760       2770       2780       2790       2800
Atsim AB236150   TAAACAAATG GAATTATAAA TTGTACATGC ATGAAAATCA TTTCCCCTGT
Atsim clone8     ---------- ---------- ---------- ---------- ----------


                 ....|....| ....|....| ....|....| ..
                         2810       2820       2830
Atsim AB236150   GTAGAAAAAA AAAAAAAAAA AAAAAAAAAA AA
Atsim clone8     ---------- ---------- ---------- --
```

Figure 7-3: Nucleotide alignment of *single minded*.

Alignment of the annotated sequence of *sim* (Akiyama-Oda and Oda, 2006) and *sim* cloned using specific primers generated complementary to the annotated sequence. Amino acids that are identical are highlighted in grey.

VI Curriculum vitae

Publikationen:

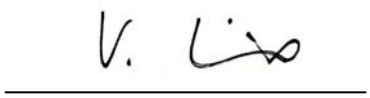
Linne, V. and Stollewerk, A. (2010). A novel function for Netrin in controlling the differentiation state of neural precursors. *Development*. Submitted.

VII Eidestattliche Erklärung

Versicherung gemäß §11, Abs.3d der Promotionsordnung

1. Ich habe die als Dissertation vorgelegte Arbeit selbst angefertigt und alle benutzten Hilfsmittel (Literatur, Apparaturen, Material) in der Arbeit angegeben.
2. Ich habe und hatte die als Dissertation vorgelegte Arbeit nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht.
3. Ich hatte weder die als Dissertation vorgelegte Arbeit noch Teile einer Abhandlung davon bei einer anderen Fakultät bzw. einem anderen Fachbereich als Dissertation eingereicht.

Mainz, den 07.09.2010



Viktoria Linne

VIII Ich bedanke mich bei