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Immunocytochemical detection of acetylated α-tubulin and *Drosophila* synapsin in the embryonic crustacean nervous system

STEFFEN HARZSCH[1,2], KLAUS ANGER[2] and RALPH R. DAWIRS[1]*

[1]*Department of Neuroanatomy, Faculty of Biology, University of Bielefeld Bielefeld and* [2]*Biologische Anstalt Helgoland, Meerestation, Helgoland, Germany*

ABSTRACT The caridean shrimp *Palaemonetes argentinus* Nobili is well suited for studying developmental aspects of the crustacean nervous system due to its rapid embryonic development and short reproductive cycle. In the present paper, we demonstrate the pattern of central axonal pathways in embryos of this species by immunohistochemical detection of acetylated α-tubulin. Development of the neuropil was elucidated by using an antibody to a *Drosophila* synapsin. In the ventral nerve cord, the segmental axonal scaffold consists of the paired lateral connectives, a median connective, and the anterior and posterior commissures. Three nerve roots were found to branch off each ganglion anlage, i.e. the main segmental nerve root, a smaller posterior nerve and the intersegmental nerve. However, this pattern is different in the mandibular segment where no intersegmental nerve and only one commissure was encountered. The anterior part of the brain consists of a tritocerebral and a deutocerebral anlage as well as the anlage of the medial protocerebrum. The latter is connected to the eyestalk via the protocerebral tract. The sequence of development of the eyestalk ganglia was demonstrated in specimens which were stained with the anti-synapsin antibody. The medulla terminalis and medulla interna are the first neuropils to appear and are still fused in early stages. Later, the medulla interna splits off the medulla terminalis. The lamina ganglionaris is the last of the eyestalk neuropils to develop. These findings prove that immunocytochemistry against acetylated α-tubulin and synapsin are valuable tools for studying the development of the crustacean nervous system.

KEY WORDS: *crustacea, embryonic development, central nervous system, synapsin, acetylated α-tubulin*

Introduction

In comparison to work performed on insects, progress in understanding the development of the crustacean nervous system has been slow. Studies in this field are hampered by difficulties in obtaining and rearing crustaceans and by the long reproductive and developmental cycles of these animals (see Helluy and Beltz, 1991; Sandeman and Sandeman, 1991; Petersen, 1995). The shrimp *Palaemonetes argentinus* Nobili (Decapopda, Caridea, Palaemonidae) is a eurhyaline species which inhabits brackish coastal lagoons (Anger *et al.*, 1994). While oogenesis has been studied histologically in this species (Goldstein and De Cidre, 1974; Schuldt, 1980), and the morphology of its larval stages has been described in some detail (Menu-Marque, 1973), embryonic development of the various organ systems is as yet unknown. In *P. argentinus* development from egg extrusion to larval hatching takes only 17 days at 24°C. Since the eggs are of a reasonable size to work with and the complete reproductive cycle can be readily accomplished in the laboratory, we chose *P. argentinus* for a series of embryological studies.

In recent years, only a small number of methods which go beyond classical histology (Scholtz, 1992; Harzsch and Dawirs, 1993, 1995/96; Helluy *et al.*, 1993, 1995, 1996; Rotllant *et al.*, 1995) has been applied to study the development of the central nervous system in crustaceans. Immunohistochemistry has been used to reveal the ontogeny of neurotransmitter and neuroendocrine systems (Beltz *et al.*, 1990, 1992; Webster and Dircksen, 1991; Helluy *et al.*, 1993; Rotllant *et al.*, 1993, 1994, 1995; Cournil *et al.*, 1995; Harzsch and Dawirs, 1995, 1996a; Schneider *et al.*, 1996). Furthermore, the neuronal expression of *engrailed* has been monitored in crustacean embryos (Patel *et al.*, 1989; Scholtz, 1995a,b). Neurogenesis has been studied by *in vitro* incorporation of BrdU in larvae but not yet in embryos (Harzsch and Dawirs, 1994, 1995, 1996b). The structure of the embryonic CNS has been investigated using neuron-specific antibodies (Dumont and Wine, 1987; Meier and Reichert, 1990; Garzino and Reichert, 1994), while axogenesis

***Address for reprints:** Department of Neuroanatomy, Faculty of Biology, University of Bielefeld, Postbox 10 01 31, D-33501 Bielefeld, Germany. FAX: 521/1065844. e-mail: R.Dawirs@biologie.uni-bielefeld.de

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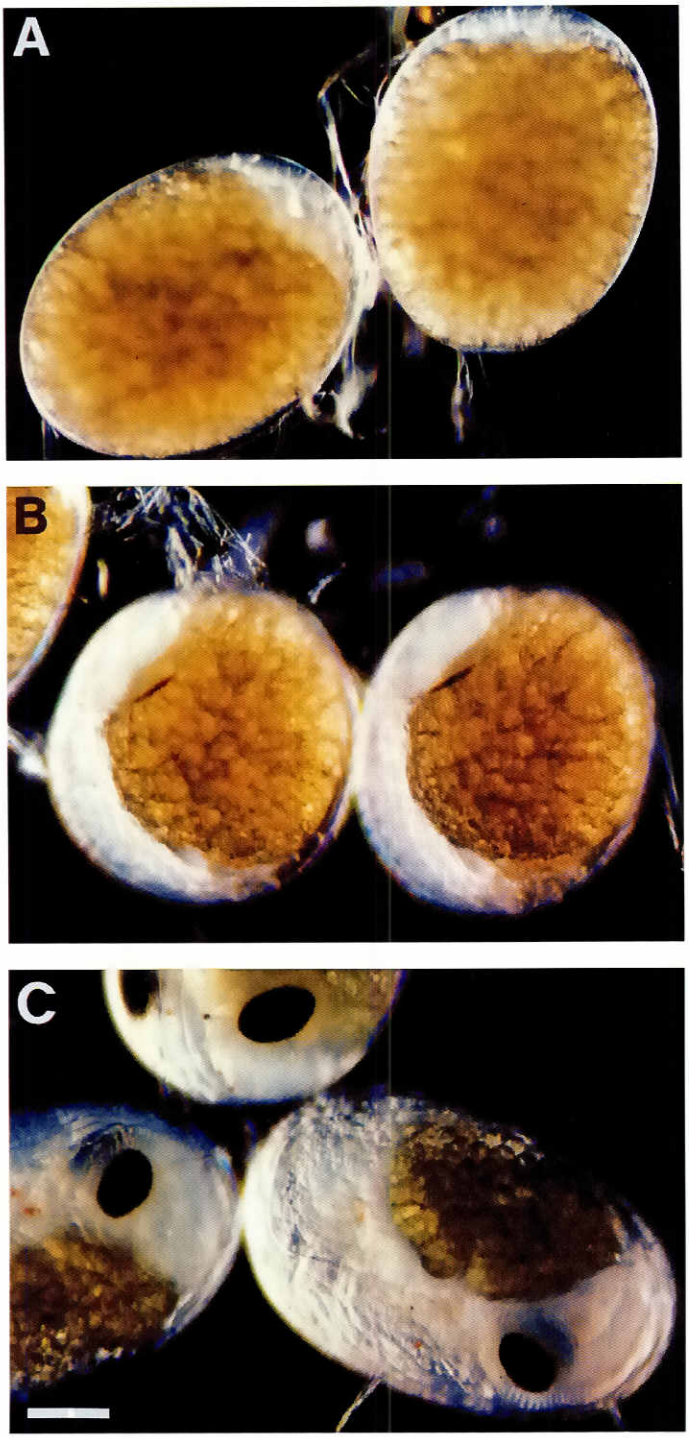

Fig. 1. Embryos of the shrimp *P. argentinus*. **(A)** *20%*, **(B)** *35%*, **(C)** *60% of development. Bar, 300 μm.*

has been visualized by rhodamine phalloidin staining (Whitington *et al.*, 1993; Scholtz, 1995a,b) and intracellular tracing techniques (Whitington *et al.*, 1993).

In the present study, we demonstrate the pattern of early axonal pathways by the immunohistochemical detection of acetylated α-tubulin. Acetylation of the side chain of lysine residues of α-tubulin is a specific posttranslational modification which increases the resistance of microtubules to depolymerization and hence may lead to microtubule stabilization (Piperno and Fuller, 1985; LeDizet and Piperno, 1991). In invertebrates, nerve cell axons are rich in acetylated α-tubulin and antibodies to this tubulin isoform have been used to stain the nervous system of, for example, nematodes (Siddiqui *et al.*, 1989; Fukushige *et al.*, 1995), insects (Wolf *et al.*, 1988; Theurkauf, 1992), molluscs (Jackson *et al.*, 1995), and echinoderms (Garcia-Arras and Viruet, 1993), but not of crustaceans.

In vertebrates, the synapsins constitute a family of presynaptic vesicle-associated phosphoproteins that are believed to participate in the regulation of neurotransmitter release (reviews by DeCamilli *et al.*, 1990; Benfenti and Valtorta, 1993; Rosahl *et al.*, 1995). Proteins which cross-react with antibodies against vertebrate synapsins are found in *Drosophila* and *Aplysia* also (Mitschulat, 1989; Bongiovi *et al.*, 1992). Recently, a *Drosophila* gene was cloned which codes for at least two inferred proteins that both contain a region with 50% amino acid identity to the highly conserved vesicle- and actin-binding "C" domain of vertebrate synapsins (Klagges *et al.*, 1996). In the present report, immunohistochemistry with the SYNORF1 antibody (Klagges *et al.*, 1996), which is directed against the *Drosophila* synapsin gene (Syn), and an antibody against acetylated α-tubulin, were used to reveal the arrangement and development of connectives, commissures, segmental and intersegmental nerves and neuropil in the embryonic ventral nerve cord and brain in *P. argentinus*.

Results

Development of the embryos

Under laboratory conditions, embryonic development in *Palaemonetes argentinus* starts immediately after extrusion of the eggs and proceeds rapidly without developmental arrest. The ovoid eggs measure approx. 1200 μm in length. At 20% development, the complete array of naupliar and postnaupliar segments is evident in the embryo which is situated on the surface of a mass of yellow yolk (Fig. 1A). At 35% development, a tiny line of pigment becomes visible in the anlage of the lateral eyes (Fig. 1B). The anlagen of the appendages are well developed by then and rhythmic contractions of the heart can be observed. During subsequent development, the pigmented zone of the eyes increases in size, the appendages grow considerably and the segmentation of the pleon can be seen from outside the egg (Fig. 1C). Meanwhile, the yolk supply shrinks and is finally depleted shortly before hatching.

Ventral nerve cord

Immunohistochemistry against acetylated α-tubulin reliably stains the axonal pathways in the embryos whereas the somata of central neurons remain unstained. Immunohistochemistry with the anti-*Drosophila* synapsin-antibody SYNORF1 results in strong staining of the developing neuropil. Synapsin-like immunoreactivity is present in some of the connectives and commissures as well while it is absent in cell somata. The developing brain is the first structure of the embryonic CNS to express anti-acetylated α-tubulin immunoreactivity and synapsin-like immunoreactivity (15% embryonic development). However, from about 20% of development onwards, axonal pathways in the ventral nerve cord are mature enough to be labeled by both methods also (Figs. 2A, 3B).

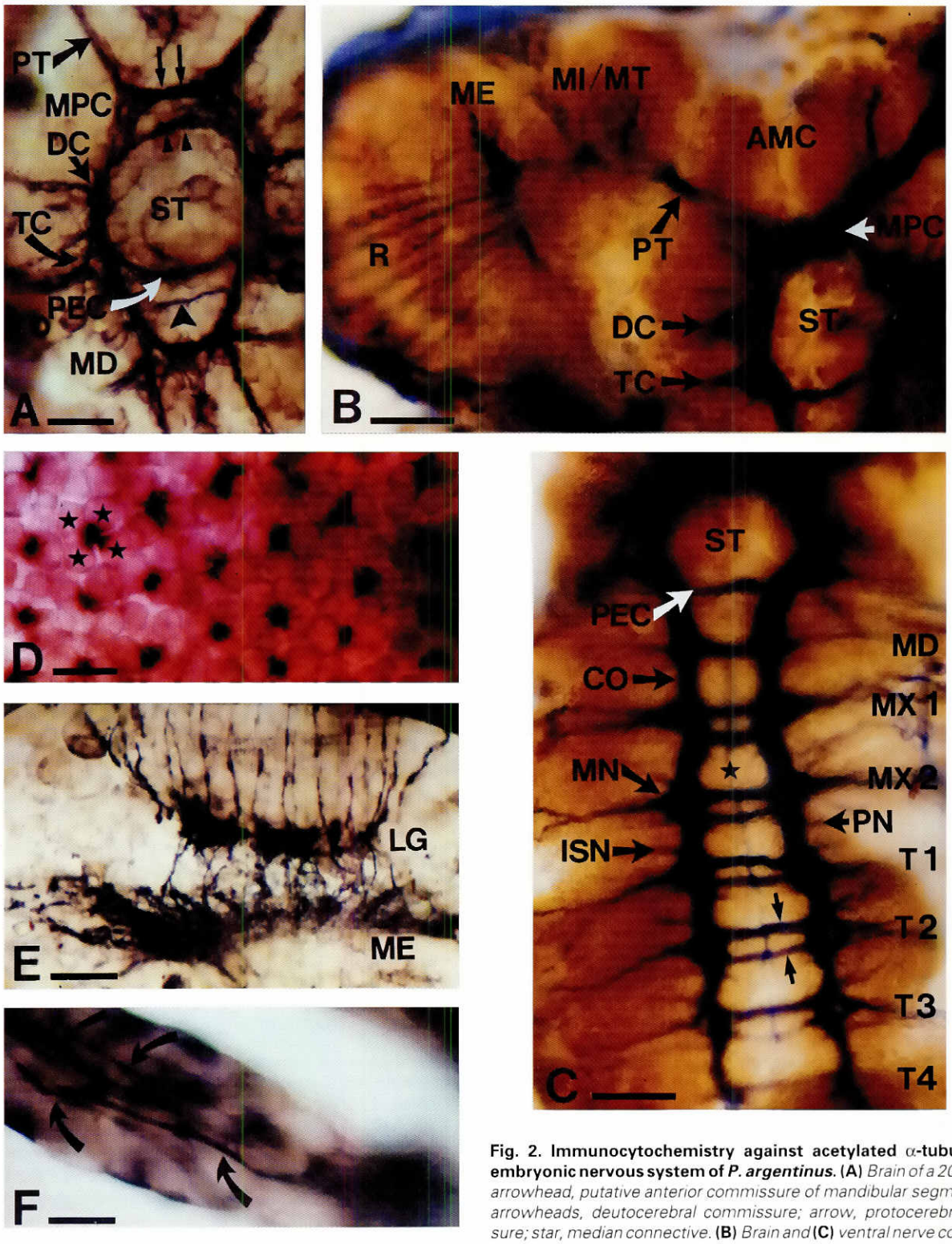

Fig. 2. Immunocytochemistry against acetylated α-tubulin in the embryonic nervous system of *P. argentinus*. **(A)** *Brain of a 20% embryo; arrowhead, putative anterior commissure of mandibular segment; double arrowheads, deutocerebral commissure; arrow, protocerebral commissure; star, median connective.* **(B)** *Brain and* **(C)** *ventral nerve cord of a 35% embryo; star, median connective; arrows, anterior and posterior commissures.* **(D)** *Retina of a 60% embryo, top view; stars indicate the 4 crystalline cone cells of a protoommatidium.* **(E)** *Retinal projections of a 50% embryo, the retina is to the top.* **(F)** *Developing leg of a 50% embryo; arrows point to nerves. Abbreviations: AMC, anterior medial cell cluster; CO, connective; DC, deutocerebrum; ISN, intersegmental nerve; LG, lamina ganglionaris; MD, mandibular segment; ME, medulla externa; MI/MT, fused medulla interna/medulla terminalis complex; MN, main segmental nerve root; MPC, medial protocerebrum; MX 1, ganglion of maxilla 1; MX 2, ganglion of maxilla 2; PEC, postesophageal commissure; PN, posterior segmental nerve root; PT, protocerebral tract; R, retina; ST, stomodaeum; TC, tritocerebrum;T 1-4, thoracic ganglia 1-4; Bars: A,D,E,F: 20 μm; B,C: 50 μm.*

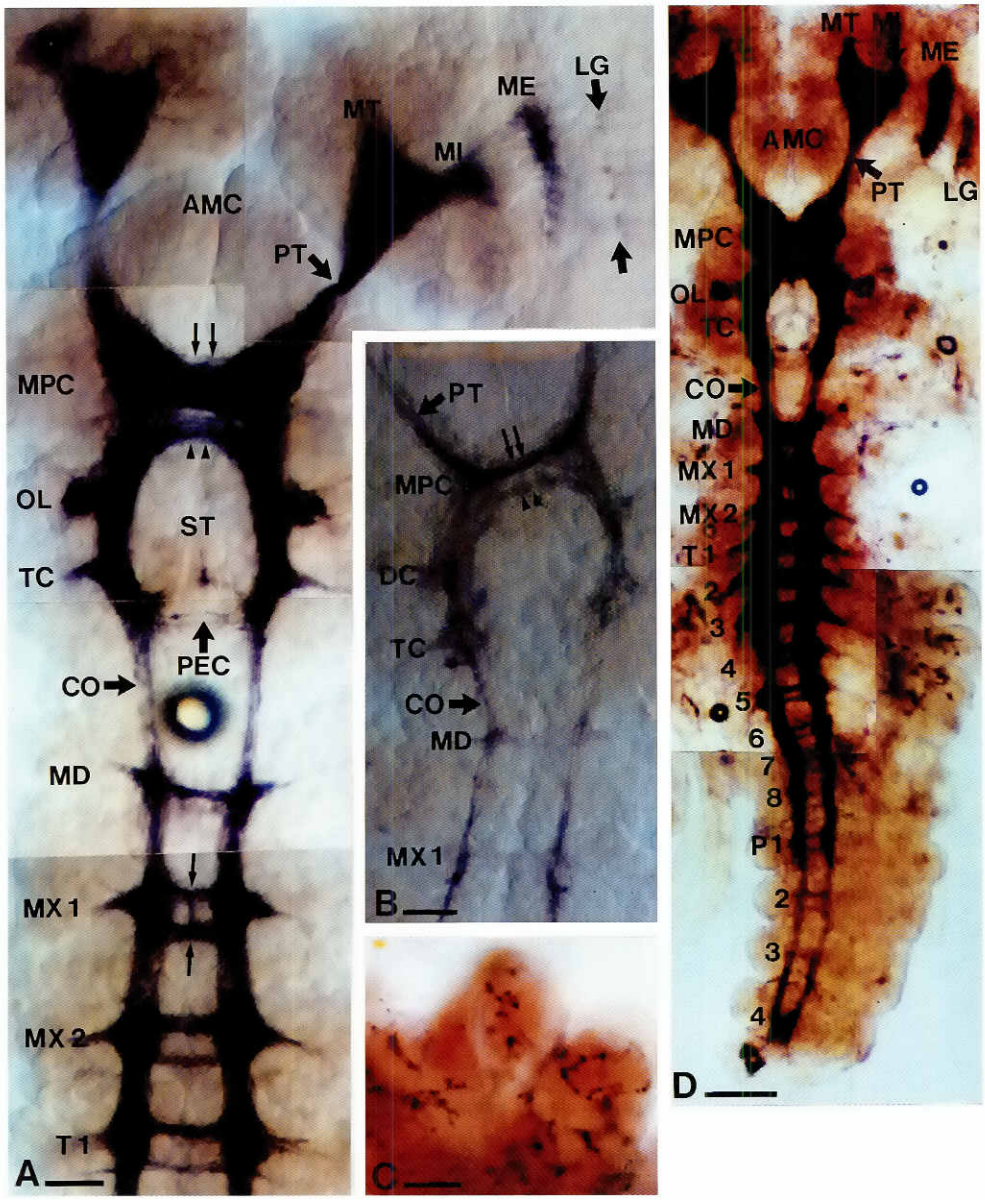

Fig. 3. Synapsin-like immunoreactivity in the embryonic nervous system of *P. argentinus*. **(A)** *40% and* **(B)** *20% embryo; single arrows, anterior and posterior commissure; double arrows, protocerebral commissure; double arrowheads, deutocerebral commissure.* **(C)** *Putative neuromuscular synaptic boutons in a 50% embryo.* **(D)** *50% embryo. Abbreviations: AMC, anterior medial cell cluster; CO, connective; DC, deutocerebrum; LG, lamina ganglionaris; MD, mandibular segment; ME, medulla externa; MI, medulla interna; MT, medulla terminalis; MPC, medial protocerebrum; MX 1, ganglion of maxilla 1; MX 2, ganglion of maxilla 2; OL, olfactory lobe; P 1-4, pleon ganglia 1-4; PEC, postesophageal commissure; PT, protocerebral tract; ST, stomodaeum; TC, tritocerebrum; T 1-8, thoracic ganglia 1-8; Bars: A,B,C: 20 µm; D: 50 µm.*

The ventral nerve cord (VNC) is characterized by a developmental gradient in which the anterior segments are more advanced while the process of formation of commissures and connectives is still under way in the more posterior segments (Figs. 2C, 3D). The gradual growth of the ganglionic neuropil in the ventral ganglia can be followed in preparations which were treated for synapsin-like immunoreactivity (Fig. 3A,B,D). In the segments of the VNC, the longitudinal tracts are interconnected by an anterior (AC) and a posterior commissure (PC, Figs. 2C, 3A). The study of the posterior segments reveals that the anterior commissure start to develop before the posterior commissure. Apart from the paired connectives (CO), another unpaired median connective (MCO) is visible, starting at the mandibular segment (MD) and running backwards (Fig. 2A,C). During development, this median connective first bridges the space between the anterior and posterior commissure before extending intersegmentally later on. The segmental nerve root (MN) leaves each ganglion anlage laterally toward the periphery, bifurcating at some distance from the ganglion. Posteriorly to this large segmental root another smaller segmental nerve root (PN) emerges from the ganglion anlagen. Halfway between the segmental commissures the intersegmental nerve root (ISN) branches off the connectives to travel laterally (Fig. 2C).

In some cases, the nerve roots which travel into the periphery can be followed until they enter the anlagen of the appendages (Fig. 2F). However, immunohistochemistry to acetylated α-tubulin does not allow a distinction between those neuronal tracts in the developing legs which originate from sensory neurons whose neurites travel centrally, and those which are composed of axons which come from central motoneurons, or both. Nevertheless, strands of synapsin-like immunoreactivity reveal that in the legs of midembryonic stages putative synaptic boutons of motor axons are present (Fig. 3C).

Medial part of the brain

While the general structural layout described for the thoracic ganglia holds for the ganglia of the maxilla 1 and maxilla 2 (MX1, 2), the pattern is different in the mandibular segment (MD). Here, no intersegmental nerve and only one commissure was found. Furthermore, synapsin-like immunoreactivity reveals that the ganglionic neuropil of the mandibular segment is much smaller than the neuropil of the following segments (Figs. 2C, 3A). The more anterior part of the brain is subdivided into an ocular-protocerebral region and the first and second antennal segments. In a posteroanterior sequence the next segmental nerve encountered anteriorly to the mandibular segment belongs to the second antenna (TC, tritocerebrum, Figs. 2A,B, 3A,B,D). The ganglion anlagen of this segment are interconnected by the postesophageal commissure

(PEC). From our preparations, we could not reliably solve the question whether this tract initially represents a "double" commissure, whether part of it is reduced or fused, or whether part of the fibers run in a bundle anterior to the stomodaeum (ST). In a 20% embryo, anti-acetylated α-tubulin immunohistochemistry reveals a small commissure between the tritocerebral commissure and the single mandibular commissure which could either represent the anterior commissure of the mandibular segment or be part of the tritocerebral commissure (Fig. 2A). The situation is similarly complicated in the segment of the first antenna (DC, deutocerebrum) which is situated anteriorly to the second antenna. While the segmental nerve and the developing olfactory lobe clearly demarcate the position and segmental origin of this part of the brain, the arrangement of commissures remains unclear (Figs. 2A,B, 3A,B,D). The deutocerebral commissure is probably fused with the most anteriorly situated commissure, that is the protocerebral commissure. In early embryonic stages, this structure is clearly composed of at least 2 distinct fiber tracts (Figs. 2A, 3B). The protocerebral tract (PT) connects the anlagen of the medial protocerebrum (MPC) to the eyestalk anlagen (Figs. 2A,B, 3A,B,D, 4A-C). A bundle of neurites emerging from the anterior medial cell cluster (AMC) enters this tract. There are distinct boundaries between the cells of the medial protocerebrum and the anlagen of the eyestalk ganglia.

Eyestalk ganglia

After entering the eyestalk anlagen, the protocerebral tract broadens and branches out to form the anlagen of the initially fused medulla interna/medulla terminalis complex (MI/MT, Figs. 2B, 4A). The sequence of development of the eyestalk neuropils can be readily analyzed in preparations which were processed for synapsin-like immunoreactivity. After the medulla interna/medulla terminalis complex, the neuropil of the medulla externa (ME) is the next to be formed (35%, Figs. 2B, 3A, 4B). An array of parallel fibers projecting into the medulla externa marks the position of the developing retina (R). These fibers emerge from clusters of immature cells within the retina and project directly into the medulla externa since the lamina ganglionaris is not present and the fibers do not form a chiasma at this stage (35%, Fig. 2B). At about 40%, the medulla interna starts to split off from the medulla terminalis (Figs. 3A, 4B). The lamina ganglionaris is the last neuropil to develop (Figs. 3A, 4C). It can first be seen as a strand of weakly synapsin-like immunoreactive material (40%) and becomes more elaborate later on (50%, Figs. 3D, 4C) The stain with the SYNORF1 antibody is less intense in the lamina ganglionaris than in the other neuropils. However, at this stage the retinal fibers still penetrate through the lamina ganglionaris to contact the medulla terminalis without forming a chiasma (Fig. 2E 50%). At this stage, the separation of medulla interna and medulla terminalis has proceeded further (Figs. 3D, 4C). At 60% development a proper retina with pigmented protoommatidia has formed and acetylated α-tubulin can be detected in the central parts of the 4 crystalline cone cells of each ommatidium (Fig. 2D).

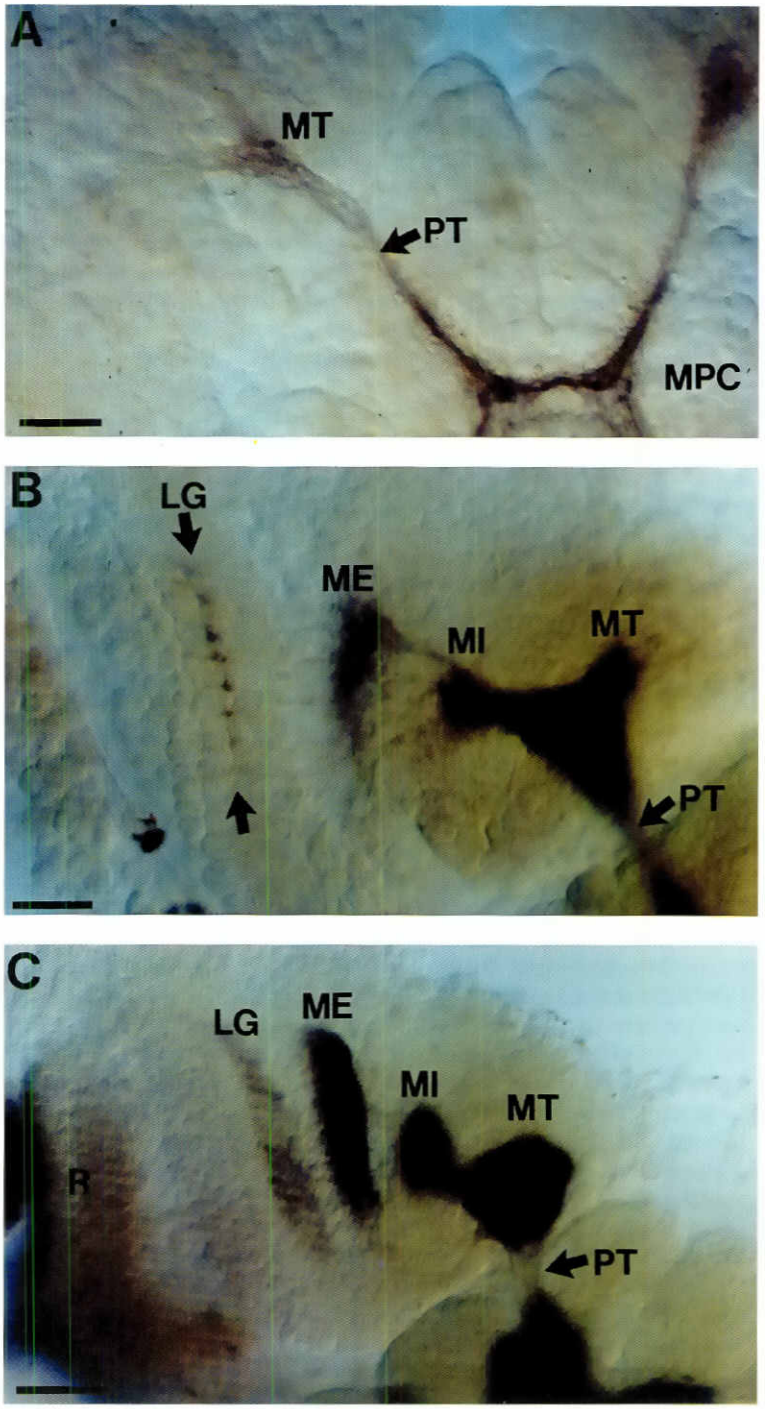

Fig. 4. Synapsin-like immunoreactivity in the developing eyestalk ganglia of *P. argentinus*. (A) *20%,* **(B)** *40% and* **(C)** *50% embryo. Abbreviations: LG, lamina ganglionaris; ME, medulla externa; MI, medulla interna; MPC, medial protocerebrum; MT, medulla terminalis; PT, protocerebral tract; R, retina. Bars: A,B: 20 μm; C: 50 μm.*

Discussion

Our present results show that the immunocytochemical detection of acetylated α-tubulin can be used to study the development of early axonal pathways in crustacean embryos at a level of

resolution which is equal or even superior to the rhodamine-phalloidin method (Whitington *et al.*, 1993; Scholtz 1995a,b). The detection of synapsin-like immunoreactivity is another tool which supplements the former method for developmental studies. The strong binding of the SYNORF1 antibody which was raised against *Drosophila* synapsin (Klagges *et al.*, 1996) suggests that proteins with a similar structure are present in the crustacean synaptic neuropil. This claim is supported by the finding that, as in *Drosophila*, the lamina ganglionaris exhibits a weaker synapsin-like immunoreactivity than the other neuropils in the embryos of *P. argentinus*. Klagges *et al.* (1996) propose that, in *Drosophila*, photoreceptors R1-R6 which have their synapses in the lamina contain no or very little of the presently known synapsin homolog isoforms. Further support for our proposition that SYNORF1 in fact binds to crustacean synaptic proteins stems from the fact that staining of putative synaptic boutons in the embryonic muscle of the thoracic appendages in *P. argentinus* resembles the structure of synaptic boutons in the larval body wall muscle and flight muscle of *Drosophila* (Klagges *et al.*, 1996).

The structure of the ganglion anlagen in the ventral nerve cord of *P. argentinus* is in good accordance with previous reports on the embryonic morphology of the ganglia in the woodlouse *Porcellio scaber*, the shrimp *Penaeus duorarum*, and the crayfish *Cherax destructor* and *Procambarus clarkii* (Elofsson, 1969; Dumont and Wine, 1987; Whitington *et al.*, 1993; Scholtz, 1995a). Accordingly, the segmental axonal scaffold in embryonic decapod crustaceans consists of the paired lateral connectives, a median connective, and the anterior and posterior commissures. Three nerve roots branch off each ganglion anlage: the main segmental nerve root, a smaller posterior nerve and the intersegmental nerve.

Our results support the findings presented in the studies of Elofsson (1969) on the shrimp *Penaeus duorarum* and Helluy *et al.* (1993) on the lobster *Homarus americanus* that neuropilar differentiation in the embryonic crustacean nervous system starts with the establishment of a nerve ring (the primordium of the brain) around the stomodaeum from which nerve roots grow towards the periphery. Furthermore, as development proceeds, the pattern of subdivision of the brain in *P. argentinus* fully confirms the concepts on head segmentation in Crustacea that have been put forward by Scholtz (1995b). In an anteroposterior sequence, an ocular-protocerebral region, the first and second antennal segment, the mandibular segment, and the first and second maxillary segments are present. However, the fact that one commissure is missing in the mandibular segment and that the size of the ganglionic neuropil is considerably reduced in this segment when compared to the more caudal ganglia has escaped notice in previous studies. This feature may be common to the decapod crustaceans since one commissure is apparently lacking in the mandibular neuromere of adult shore crabs *Carcinus maenas* (Plachta and Dircksen, personal communication), embryonic crayfish *Cherax destructor* (Scholtz, personal communication) and embryonic spider crabs *Hyas araneus* (Harzsch and Dawirs, unpublished results) as well. Possible explanations are that a fusion of the anterior and posterior commissures has taken place, or one of these has been reduced. Another possibility is that the anterior commissure is represented by the rudimentary commissural fiber bundle which was found behind the subesophageal commissure in some preparations and that the "missing part" of the mandibular neuropil has moved anteriorly to adjoin the tritocerebral neuropil. However, these interpretations leave open the question whether the postesophageal commissure, interconnecting the two halves of the tritocerebrum, develops from a double commissure or whether part of this tritocerebral commissure runs anteriorly to the stomodaeum. The deutocerebral commissure probably runs anteriorly to the stomodaeum, fused with the protocerebral commissure. The question whether these two tracts initially represent double commissures has to be left open to future studies.

By studying the pattern of synapsin-like immunoreactivity, we were able to follow the sequence of generation of the embryonic eyestalk neuropils in *P. argentinus*. The medulla terminalis is the first neuropil to develop. The medulla interna is eventually split off this neuropile. The medulla externa is the next neuropil which expresses synapsin-like immunoreactivity while the lamina ganglionaris is the last of the eyestalk neuropils to be formed. This sequence of development is in good accordance with Elofsson's (1969) data on the embryonic formation of the optic system of the shrimp *P. duorarum*. Furthermore, our results fully support the hypothesis put forward by Elofsson (1969) and Elofsson and Dahl (1970) that the medulla interna is split off the medulla terminalis and that these two neuropils were initially part of the medial protocerebrum. However, we found little evidence that the anlagen of the lamina ganglionaris and the medulla externa are situated perpendicular to each other. Elofsson and Dahl (1970) propose that this is a fundamental feature which is common to all developing crustacean compound eyes. Furthermore, it is difficult to interpret our findings according to Elofsson and Dahl's (1970) suggestion about the formation of the optic chiasmata by a rotation of the medulla externa. In *P. argentinus*, up until midembryonic stages at least part of the fibers which originate in the retina transverse the anlage of the medulla terminalis to terminate in the developing medulla externa without forming any obvious chiasma. However, it might be that the chiasma is formed later by the outgrowing axons of interneurons. Hence, development of optic anlagen in crustaceans remains a controversial issue. Future efforts should be directed towards understanding the segmental origin and development of the ocular-protocerebral region and unraveling the arrangement of commissures in the crustacean brain. Due to its rapid embryonic development and short reproductive cycle we consider *P. argentinus* a valuable model organism to address these questions.

Materials and Methods

Adult females of *Palaemonetes argentinus* Nobili were obtained from freshwater creeks adjacent to the Laguna Mar Chiquita, a brackish coastal lagoon in Argentina (Anger *et al.*, 1994), and transported to the Helgoland Marine Biological Station (Germany). In the laboratory, they were maintained at a constant 24°C and a salinity of 1‰ which is close to the conditions in the natural habitat during the reproductive season (November-February). Embryonic development from egg extrusion to larval hatching lasts 17 days under these conditions. The embryos were staged on the basis of developmental time. An embryo which has gone through, for example, 50% of its embryonic development is referred to as a "50% embryo" in this text.

Embryos were dissected in the rearing medium and then fixed for 4 h in 4% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.4) at room temperature. After fixation, specimens were washed in several changes of phosphate buffered saline (PBS) for 1 h and then preincubated in PBS containing 5% normal goat serum and 0.3% Triton X-100 (PBS-TX) at room temperature for 1 h. Incubation in the anti-acetylated α-tubulin antibody (Sigma, 1:100 in PBS-TX) and the SYNORF1 antibody (1:30, Klagges *et al.*, 1996, antibody provided by Prof. Dr. E. Buchner, Würzburg, Germany)

was carried out overnight at 4°C. There was no neuronal staining in specificity controls in which the primary antibody was omitted. After washing in PBS for 2 h, specimens were incubated in an alkaline phosphatase conjugated goat-anti-mouse antibody (Sigma) for 3 h at room temperature. The label was developed using Sigma Fast BCIP/NBT substrate tablets (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium, Sigma). The specimens were either mounted in glycerol or dehydrated and mounted in Eukitt.

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