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SEROTONIN IMMUNOREACTIVITY IN THE VENTRAL NERVE CORD OF THE PRIMITIVE CRUSTACEAN *ANASPIDES TASMANIAE* CLOSELY RESEMBLES THAT OF CRAYFISH

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Summary

Syncarid crustaceans, of which only a few living species remain, have articulated segments with well-developed appendages along the length of the body, an arrangement thought to resemble that of the earliest malacostracan crustaceans. Decapod malacostracans have fused thoracic segments and reduced abdominal appendages. Modern representatives of the two groups are separated by at least 300 million years of evolutionary history. The serotonin immunoreactivity of ganglia and connectives from the ventral nerve cord of the syncarid *Anaspides tasmaniae* was compared with that of serially homologous ganglia of the crayfish *Cherax destructor*. Both species show the serotonin-immunoreactive longitudinal fibre bundles described from other decapods and thought to be part of a neuromodulatory network. They also have in common a number of the cell bodies associated with this system. Each species has some serotonergic cells in the region examined that are not present, or that do not stain, in the other species.

Key words: serotonin, crayfish, *Anaspides tasmaniae*, homology, neural evolution.

Introduction

Natural selection operates on both morphology and behaviour. Whether there are patterns to the morphological and chemical changes in neurones during behavioural evolution is not known, and there is no assistance from the fossil record at the neuronal level. Comparisons of closely related species are providing evidence of an underlying conservatism in a number of aspects of neural structure (Northcutt, 1984; Meier *et al.* 1991; Paul, 1989, 1991; Katz, 1991). More examples are needed, however, because although circuits and their components do change, it is not yet clear which features are relatively well conserved and which are modified.

One difficulty of using the comparative method in an evolutionary context is deriving an acceptable representation of the primitive condition by evaluating a number of related, but differently evolved, modern species. We are testing a novel approach to this problem, open to us because Australia has a relict population of the syncarid crustacean *Anaspides tasmaniae*. Fossil and morphological evidence from extant syncarids suggests that they have altered little in their external morphology for at least 300 million years (Schram, 1983) and probably represent a form close to the ancestral malacostracan condition. If constancy in external morphology is mirrored by a similar stability in the nervous system, a likely but unproved supposition, then we may have access to something resembling an ancestral malacostracan nervous system. Even if that hypothesis eventually proves untenable, comparisons between aspects of the nervous system of *Anaspides tasmaniae* and a decapod should be informative because of the length of time since their evolutionary separation. The Australian crayfish *Cherax destructor* was used for the comparison because of the rapidly accumulating body of information concerning its behaviour and the structure and function of its nervous system (e.g. Macmillan and Field, 1994; Pasztor and Macmillan, 1990; Sandeman *et al.* 1988, 1993). Here we report on a comparison of serotonergic immunoreactivity in the neurones of the anterior nerve cord of *Anaspides tasmaniae* and *Cherax destructor*.

Materials and methods

The malacostracan central nervous system consists of a dorsal, cerebral ganglion that gives rise posteriorly to a pair of circumoesophageal connectives leading to a ventral nerve cord composed of a series of ganglia joined by paired connectives. There are 18 primordial neuromeres in the nerve cord of malacostracans, but they aggregate into ganglia in different

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ways depending on the species. Macruran decapods, such as crayfish and lobsters, have six abdominal ganglia and five thoracic ganglia associated with the walking legs. *Anaspides tasmaniae* lacks a carapace and the segments of the thorax are articulated, but the serial homology of its segments and neuromeres relative to those of *Cherax destructor* is clear (E. Wallis, in preparation). We have used the crayfish numbering system for ease of comparison: T1–T5 for the ganglia associated with the five posterior appendages of the thorax, and A1–A6 for the six abdominal segments.

Immature specimens (0.30–0.45 g; 24–30 mm carapace length) of *Cherax destructo*r were taken from a population cultured in our laboratory from stock obtained through a local distributor. Adult *Anaspides tasmaniae* (0.25–0.50 g, 23–35 mm total length) were collected from Mount Wellington, Tasmania, and maintained at 4 °C in circulating mountain water. *C. destructor* were anaesthetised by cooling on ice and *A. tasmaniae* by exposure to a nitrogen atmosphere. Animals were pinned out in a bath lined with Sylgard 184 (Dow Corning) and the ventral nerve cords dissected at 4 °C in crayfish saline solution containing (in $mmol\,l^{-1}$): 205 NaCl, 5.4 KCl, 13.5 $CaCl_2$, 2.6 $MgCl_2$, 10 Tris, 5 maleic acid, pH 7.4. The dissected preparations were pinned out in fresh saline and all non-neural tissue was carefully removed prior to fixation for 24 h at room temperature in 4% formaldehyde in $0.1\,mol\,l^{-1}$ phosphate buffer, pH 7.0. Following fixation, the tissue was rinsed with phosphate-buffered saline (PBS) and subjected to the following series of incubations to increase antibody penetration: 70% ethanol for 30 min at room temperature; several changes of PBS-0.3 (0.05% thimerosal and 0.3% Triton X-100, pH 7.0, in PBS) over 10–15 h.

The tissues were then pre-incubated in normal goat serum (NGS) diluted 1:10 in PBS-0.3 for 6–8 h, followed by a 15–20 h incubation in rabbit anti-serotonin antiserum (antiserum 196C, Costa *et al.* 1982) diluted 1:1000 in PBS-0.3 containing 10% NGS. Tissue was then rinsed for 6–8 h in PBS-0.3, incubated in biotinylated goat anti-rabbit secondary antiserum (Vector Laboratories Inc.) for 15–20 h, rinsed for 6–8 h, incubated at 4 °C in avidin–biotin–peroxidase complex (ABC; Vector Laboratories, Inc.) for 15–20 h, and rinsed in PBS-0.3 for 6–8 h. After this, the nerve cords were first incubated in a solution containing (in $g\,ml^{-1}$) 6.0 Tris, 6.0 NaCl and 0.5 ammonium nickel sulphate for 15–20 h and then incubated in diaminobenzidine-tetrahydrochloride (DAB) at a concentration of 0.02% in Tris buffer for 5 min at room temperature prior to transferring them to fresh DAB solution containing 0.005% H_2O_2. The peroxidase reaction was observed and stopped, usually after 2–3 min, by transferring tissue to PBS-0.3. Tissue was then mounted on microscope slides in phosphate-buffered glycerol. The specificity of the staining was tested by pre-adsorbing diluted antiserum for 24 h with 10^{-3} or $10^{-2}\,mol\,l^{-1}$ serotonin. In both *C. destructor* and *A. tasmaniae*, $10^{-3}\,mol\,l^{-1}$ serotonin caused a reduction in the staining of the cell bodies, nerve terminals and axons, and $10^{-2}\,mol\,l^{-1}$ serotonin abolished the staining of the nerve terminals and dramatically reduced the staining of the cell bodies and axons. The high concentration of serotonin necessary to suppress the staining is probably due to the fact that the antibody is raised against serotonin coupled to a carrier protein with formaldehyde and recognises the conjugate better than serotonin alone. No staining was observed when the serotonin antiserum was omitted. Six *A. tasmaniae* and nine *C. destructor* nerve cords were processed.

Results

A comparison of the prepared ventral nerve cords revealed a network of labelled, serotonin-immunoreactive structures in both *C. destructor* and *A. tasmaniae*. These included nerve cell bodies, axons, branching fibres and neuropilar regions within the central nervous system (CNS) and arborisations of varicose fibres in the the nerve roots. An example of the immunoreactive neurones in an *A. tasmaniae* preparation is shown on the cover of this issue of the journal and *camera lucida* reconstructions of the stained structures in the entire thoracic cord of both animals are shown in Fig. 1.

Cherax destructor

Sixteen clearly labelled cell bodies were consistently detected in the last five thoracic and first abdominal ganglia of *C. destructor*. These elements are essentially the same as those previously described in the same cord regions of *Homarus americanus* (Beltz and Kravitz, 1983) and *Procambarus clarkii* (Real and Czternasty, 1990). Three distinct, longitudinal fibre bundles run the entire length of the thoracic cord on either side (Fig. 1A), laterally (lateral fibre bundle, LFB), in the midline (medial fibre bundle, MFB) and between these two (central fibre bundle, CFB). As in the other species studied, the positions of the cell bodies and axons are characteristic of the segment. A pair of postero-lateral cell bodies stains in each of the first four segments (T1–T4), and each cell body gives rise to an axon crossing the midline to run anteriorly in the contralateral CFB. In T5, the cell bodies are medial and their axons run into the ipsilateral MFB. This pattern is also followed by a pair of cells in the ganglion of the first abdominal segment (A1), which contains, in addition, four small immunoreactive cells. The close similarity in the organizaton of serotonergic neurones from representatives of the three astacid families, Nephropidsidae (*Homarus*), Astacidae (*Procambarus*) and the Gondwanan Parastacidae (*Cherax*), provides further evidence of the morphological constancy exhibited by this presumptive serotoninergic secretory system.

Anaspides tasmaniae

In overall disposition, the distribution of serotonin immunoreactivity in the *A. tasmaniae* ventral nerve cord was remarkably similar to that described above for *C. destructor*. Three longitudinal bundles can be distinguished clearly on the basis of their position in the cord (Fig. 1B). Varicose branchings in the nerve roots are also present but less pronounced than in *C. destructor*. A pair of cells with axons crossing to the contralateral CFB stains in segments T1, T3 and

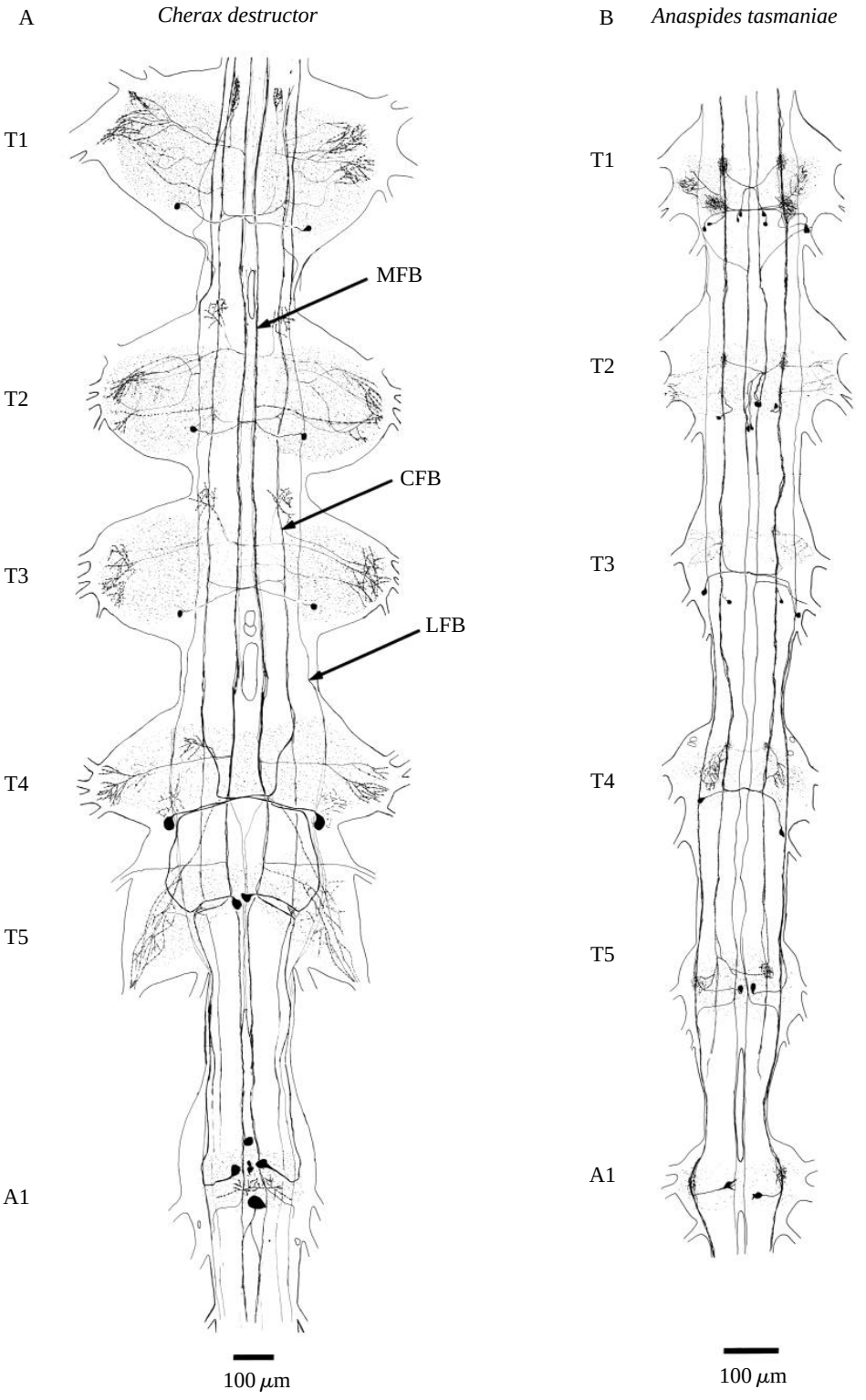

Fig. 1. *Camera lucida* drawings of structures showing serotonin immunoreactivity in the anterior ventral nerve cords of the crayfish *Cherax destructor* and the primitive syncarid malacostracan *Anaspides tasmaniae*. Ventral cords are drawn to the same scale, but parts of the *Cherax* connectives have been omitted to emphasise the similarities between the ganglia of the two animals. T1–T5, thoracic ganglia; A1, abdominal segment 1. MFB, CFB and LFB refer to medial, central and lateral fibre bundles, respectively.

T4, but is missing from segment T2, a conclusion we checked carefully with additional replications. Segments T5 and A1 also contain medially located cell bodies with axons to the ipsilateral MFB. On the basis of neuromere, neuronal morphology, position within the ganglia and projection in both ganglia and nerve cord, we propose that these neurones are homologues of those described in *C. destructor* and other malacostracans. That is to say, we propose that the neurones have the same embryological and evolutionary antecedents.

There were, however, a number of interesting differences. Additional, medial immunoreactive cells in A1, a feature found in *C. destuctor* and the other decapod species studied, are lacking in *A. tasmaniae*. There is also the apparent deletion in T2 mentioned above, for which we see no obvious explanation

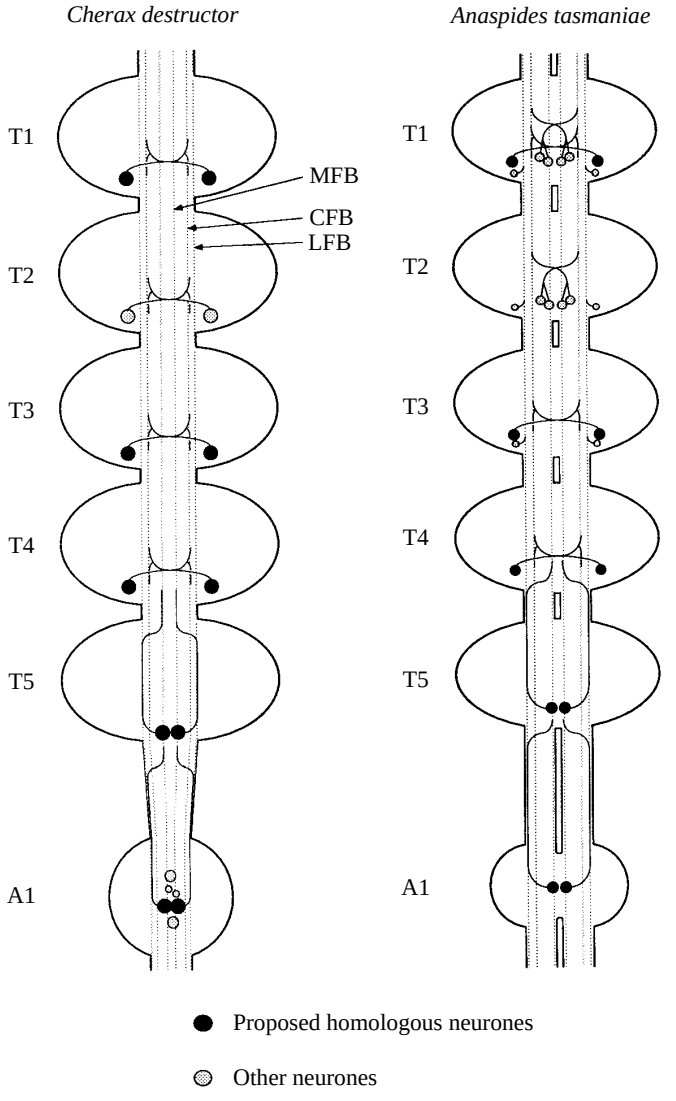

Fig. 2. Diagrammatic comparison of neurones and longitudinal bundles that stain for serotonin in the anterior ventral nerve cord of *C. destructor* and *A. tasmaniae*. Proposed homologous neurones are shown in black; those staining in only one of the species are shown in grey. MFB, CFB and LFB refer to medial, central and lateral fibre bundles, respectively.

in the morphology of the animal. In contrast, a number of cells stained that are not present in the other species, but which are associated with the longitudinal network of serotonin-immunoreactive tracts in *A. tasmaniae* (Fig. 1B). Segments T1–T3 each show a bilateral pair of cells posteriorly, near the lateral boundary of the ganglion, which each give rise to a fine process joining the ipsilateral CFB. Segments T1 and T2 also have a pair of postero-medial cell bodies on either side giving rise to axons running together towards the contralateral CFB, near which they terminate in a tuft of fine arborisations (Fig. 1B) rather than contributing an axon to the bundle itself. The results of the comparison are summarised in diagrammatic form in Fig. 2.

Discussion

The most significant finding of the study is the striking similarity of the serotonergic fibre bundles of the ventral nerve cords, and of some of the neurones in the segmental ganglia contributing axons to them, in the two animals. Not only are the species separated by at least 300 million years of evolutionary history, but the morphology of the trunk segments of the crayfish has diverged markedly from the primitive condition, more closely represented by *A. tasmaniae*. The anterior trunk segments of the syncarid are all articulated and have essentially the same morphology, whereas those of the crayfish are fused and variously modified to contribute to the rigid cephalothorax. Is the conservatism revealed here general or is it a property of certain types of neural systems? There is evidence that the serotonergic system functions in a neuromodulatory capacity in crustaceans (Beltz and Kravitz, 1986; Harris-Warrick and Kravitz, 1984) and it is possible that its apparently constant elements are related to this function. A modulatory system could perhaps be expected to operate with a certain degree of independence from detailed modifications in coordinating networks downstream of its input. In *A. tasmaniae*, as in the other macruran species, the longitudinally staining serotonergic immunoreactive tracts run the length of the abdomen, but no cell bodies stain. We have not resolved the situation in ganglia anterior to the ones reported on here because they are too small for reliable application of the whole-mount technique we used and will require analysis by histological sectioning.

The differences revealed by the serotonin immunoreactivity in the two animals raises a number of questions. Both animals have at least some neurones that are not represented in the other, and there are three possible mechanisms which alone, or in combination, could explain this finding. The first is that unique cells could be additions since the evolutionary divergence of the groups; the second is that the absence of neurones could represent deletions; the third is that homologous neurones are present in both species, but no longer stain for serotonin in one of them (Brauth, 1990). Our present results do not permit us to distinguish between these possibilities, although a number of authors have argued against the second mechanism as a major contributor to neural evolution, particularly where the changes in somatic morphology are not extreme or where CNS structures are involved (Kavanau, 1990). There are, however, numerous examples of neuronal loss in related groups, so it remains a possibility until specifically excluded. Objective consideration of cell loss is somewhat confounded by the third possibility, that morphologically similar circuits can change function under the influence of neuromodulatory changes (Katz, 1991) and that apparently homologous cells can change their transmitter chemistry (Katz and Tazaki, 1992). Only a combination of detailed analyses of cellular properties in sharply defined groups of cells and comparisons across related species will permit us to determine which of the mechanisms operate during evolution and under what circumstances.

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