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Neuronal Pathways of Classical Crustacean Neurohormones in the Central Nervous System of the Woodlouse, Oniscus asellus (L.)

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Neuronal pathways of classical crustacean neurohormones in the central nervous system of the woodlouse, *Oniscus asellus* (L.)

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CONTENTS

LIST OF ABBREVIATIONS USED IN TEXT AND FIGURES

A1–A6	first to sixth abdominal neuromer
AG	abdominal ganglia complex
Arv-CHH	*Armadillidium vulgare* CHH*
Cam-CHH	*Carcinus maenas* CHH*
Cam-MIH	*Carcinus maenas* MIH*
cb	central body (corpus centrale)
cf	commissural fibre
CHH	crustacean hyperglycemic hormone
CNS	central nervous system
col	collaterals
DC	deutocerebrum
GAR	goat anti-rabbit serum
HPLC	high pressure liquid chromatography
LCNP	lateral cephalic nerve plexus
ldf	lateral dorsal fibre
LG	lamina ganglionaris
LO	optic lobe (lobus opticus)
MD	mandibular ganglion
mdf	median dorsal fibre
ME	medulla externa
MI	medulla interna
MIH	moult inhibiting hormone
mp	median plexus
MT	medulla terminalis
MX1	first maxillary ganglion
MX2	second maxillary ganglion
MXP	maxillipedal ganglion (MXP = T1)
NSC	neurosecretory cells
NO	optic nerve (nervus opticus)
PAP	peroxidase–antiperoxidase complex
Pod-CHH	*Porcellio dilatatus* VIH*
PDH	pigment dispersing hormone
PGR	PDH-immunoreactive group of neurons
PRC	protocerebrum
RPCH	red pigment concentrating hormone
RGR	RPCH-immunoreactive group of neurons
SG	sinus gland
SM	somatic muscle
T1–T8	first to eighth thoracic ganglion
TC	tritocerebrum
TG	thoracic ganglia
VIH	vitellogenesis inhibiting hormone
VNC	ventral nerve cord
YO	Y-organ

Anatomical terminology adopted from Walker (1935).

* Peptide nomenclature according to Raina & Gäde (1988).

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SUMMARY

Neuropeptide-immunoreactive neurons have been mapped by immunocytochemistry in whole-mount preparations and sections of the central nervous system of *Oniscus asellus*. We tested rabbit antisera against decapod crustacean hyperglycemic hormone (CHH), moult inhibiting hormone (MIH), pigment dispersing hormone (PDH) and red pigment concentrating hormone (RPCH). Four CHH- and three PDH-immunoreactive neurons localized in the superior median protocerebrum of the brain constitute neurosecretory pathways to the neurohaemal sinus gland. No immunoreactive structures have been detected with an antiserum against MIH of *Carcinus maenus*. Another, newly identified neurosecretory pathway is formed by a group of RPCH-immunoreactive neurons in the mandibular ganglion. These neurons project to the neurohaemal lateral cephalic nerve plexus. Further PDH- and RPCH-immunoreactive neurons and fibres occur in the brain and the ventral nerve cord (VNC). Two groups of PDH-immunoreactive neurons supply brain and optic lobe neuropils, the bases of the ommatidia, and probably give rise to descending fibres innervating all VNC-neuropils. Two groups and five individuals of RPCH-immunoreactive neurons that innervate several brain neuropils or occur as ascending neurons in the VNC have been reconstructed. The CHH-immunoreactive neurons, and distinct types of PDH- and RPCH-immunoreactive neurons obviously belong to classical hormone-producing neurosecretory pathways. At least the CHH-immunoreactive cells seem to be part of an isopod homologue of the decapod X-organ. The existence of other PDH- and RPCH-immunoreactive interneurons suggests additional functions of these peptides as neurotransmitters or neuromodulators, which is in agreement with similar observations in the decapod central nervous system.

1. INTRODUCTION

In the last two decades our knowledge of neuroendocrine regulatory mechanisms in decapod crustaceans has increased considerably (for review see Cooke & Sullivan 1982; Webster & Keller 1987; Keller 1992). Another malacostracan order, the isopods, has received less attention when the efforts to completely characterize various crustacean neurohormones biochemically and to identify their cellular sources are considered (Martin 1988), although isopod species have proved to be convenient model systems for neurophysiological studies of neuroendocrine control mechanisms (Steel 1980, 1982; Chaigneau 1983; Chiang & Steel 1984).

In their pioneering work, Gersch & Eibisch (1976) demonstrated the existence of a hyperglycemic factor in the isopod *Armadillidium vulgare* by ablation of the sinus gland (SG) and other parts of the brain. Evidence for the existence of a hormone related to decapod crustacean hyperglycemic hormones (CHHs) has later been provided in isopods by studies with radioimmunoassays and biotests (Jaros & Reichwein-Roderburg 1981; Leuven *et al.* 1982; Keller *et al.* 1985). Martin *et al.* (1984*a*) reported the first isolation and characterization of a CHH from *Porcellio dilatatus*, and, more recently, Martin *et al.* (1993) succeeded in the complete molecular characterization of a CHH of 73 amino-acids isolated from sinus glands of the pillbug *Armadillidium vulgare* (Arv-CHH according to the nomenclature of Raina & Gäde (1988)). This peptide shares sequence and disulphide bridge homologies with representatives of a family of decapod peptides that are known to display hyperglycemic effects and/or moult inhibiting or vitellogenesis inhibiting biological activities (for recent review see Keller 1992).

Martin *et al.* (1984*b*) have demonstrated protocerebral cells and a pathway into the SG of *Porcellio dilatatus* that are immunoreactive to an antiserum raised against crude extracts of the SG of *Carcinus maenas*. This antiserum supposedly contained CHH-specific antibodies as a major component that cross-reacted with the Pod-CHH. This finding further indicated that, due to their striking sequence homologies, the 'true' CHH forms (Kegel *et al.* 1989, 1991; Chang *et al.* 1990; Tensen *et al.* 1989, 1991; Martin *et al.* 1993) may readily cross-react with different anti-CHH sera used in several comparative studies on decapod crustaceans (Jaros & Reichwein-Roderburg 1981; Gorgels-Kallen *et al.* 1982; Leuven *et al.* 1982; Mangerich *et al.* 1986; Dircksen *et al.* 1988; Dircksen 1992). The situation is less evident in terms of putative sequence homologies for another SG hormone considered as a further member of the above-mentioned decapod peptide family, the moult inhibiting hormone (MIH). This peptide was identified from SG extracts of *Carcinus maenas* as a potent inhibitor of the ecdysteroid production of the moulting glands (Cam-MIH; Webster 1991). Compared with astacuran CHH-like molecules which showed moult inhibiting activity (Chang *et al.* 1990), Cam-MIH did not display any hyperglycemic activity (Webster & Keller 1986). Antisera against this peptide have detected MIH-immunoreactive neuronal structures in different brachyuran species (Dircksen *et al.* 1988; Dircksen 1992) but not in astacuran species (H. Dircksen & S.G. Webster, unpublished results). Unfortunately, information on the occurrence and nature of identified MIH-like molecules in isopods is completely lacking.

Chromatophorotropic factors and their effects on integument colour change and/or eye pigment movements have been the subject of numerous studies in several decapod and a few marine isopod species (Stahl 1938*a*,*b*; Kleinholz 1937, 1961; Fingerman 1969, 1970; Castrucci & Mendes 1975; Rao 1985). Experiments with interspecies injections of diverse ganglia extracts led to the assumption of general pigmentary effectors in several malacostracan crustaceans. However, the

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results could not as yet be combined into a common principle of action with regard to effector specificity, namely of the pigment concentrating or dispersing factors acting on different types of responding chromatophores (Kleinholz 1937; Stahl 1938*a*,*b*; Okay 1945; Suneson 1946; McWhinnie & Sweeney 1955). However, it became clear from biochemical studies on several decapod species that all isolated red pigment concentrating hormones (RPCH) have the same primary structure (Gaus *et al.* 1990), whereas the identified pigment dispersing hormones (PDHs) are not identical but homologous to a high degree (Rao *et al.* 1985; Kleinholz *et al.* 1986; Rao & Riehm 1988, 1989). To this date, only a few decapod species have been examined in some detail by use of immunochemical methods with regard to the cellular sources of RPCH and PDH (Bellon-Humbert *et al.* 1986; Mangerich *et al.* 1986, 1987; Dircksen *et al.* 1987; Mangerich & Keller 1988; Sherff & Mulloney 1991). Castrucci & Mendes (1975) were the last authors who clearly demonstrated that not only marine but also terrestrial isopod species have at least pigment dispersing factors, although a colour change behaviour was not evident in these particular species. Up to the present date, there is nothing known about the biochemical nature of these factors and their possible cellular origin in isopods.

The first description of four types of neurosecretory cells (NSC classified as A, B, β, γ) in an isopod, *Armadillidium vulgare*, by Matsumoto (1959) stimulated similar studies in several other isopod species by use of conventional staining methods for neurosecretion (see table 1 in Demassieux 1979). Messner (1966) was the first to describe NSC in the brain of the terrestrial isopod *Oniscus asellus* some of which project into the SG. Re-examination of the SG-innervating neurosecretory systems by use of cobalt backfilling methods, electron microscopy and electrophysiology later provided the most detailed description of the fine structural organization and branching patterns of these particular NSC (Chiang & Steel 1984, 1985*a*,*b*, 1986). Several other authors adopted Matsumoto's (1959) NSC terminology for the description of further neurosecretory pathways from the mouthpart ganglia to the peripheral so-called lateral cephalic nerve plexus (LCNP Messner 1963; Besse & Legrand 1964) close to the moulting gland (Y-organ) of oniscoid species and provided evidence for a neurohaemal nature of this structure (see Maissiat *et al.* 1979; Martin *et al.* 1983; Martin 1988). Based upon cross-reactivities of antisera against the classical decapod hormones CHH, MIH, PDH and RPCH with putatively similar isopod neurohormones, we have aimed at a comprehensive immunocytochemical approach to identify the cellular sources of these substances in *Oniscus asellus*. We have not only examined the well established NSC but tried to elucidate the complete pathways of all immunoreactive NSC and other neurons in the brain and the VNC of this species. Moreover, we have tried to address the question of whether the principal organization of identifiable neurosecretory neurons and their neurohaemal release sites in this isopod are similar or perhaps homologous to the well known neurosecretory systems of decapods.

2. MATERIALS AND METHODS

(*a*) *Animals and tissue preparation*

Adult woodlice, *Oniscus asellus*, were collected in the field and maintained in colonies at 22 °C under a 12 h:12 h light:dark illumination régime. Animals of both sexes in the intermoult stage (stage 15, Steel 1982) were chosen. Dissection of ganglia was done under an ice-chilled isopod saline (pH 7.9) prepared according to Martin *et al.* (1984*b*).

For whole-mount preparations, 2% sodium phosphate buffered paraformaldehyde containing 15% saturated aqueous picric acid (pH 7.4; Stefanini *et al.* 1967) was applied for 4–24 h at 4 °C. Some specimens were fixed in 0.1 M sodium phosphate buffered 4% paraformaldehyde containing 1% EDC (1-ethyl-3,3′-dimethylaminopropylcarbodiimide) and 0.9% NaCl according to Dircksen & Keller (1988).

For paraplast embedding, dissected tissues were fixed immediately in Bouin's solution for 2–4 h at room temperature or overnight at 4 °C. In addition, Bouin's solution without acetic acid and a fixative described by Boer *et al.* (1979) were tested. However, these last two fixatives resulted in weak immunostainings in most cases. For longitudinal and cross-sectioning of the whole anterior body up to the mouthpart ganglia some animals with soft cuticle (ecdysial or early postmoult stages 12–13 according to Steel (1982)) were fixed in Bouin's solution and embedded in Paraplast.

(*b*) *Immunocytochemical techniques*

Whole-mount preparations of fixed ganglia were washed first with isotonic buffer (0.01 M sodium phosphate buffered saline (PBS) containing 0.52 M sucrose) and subsequently treated with PBS containing 0.5% Triton X-100 (PTX) to facilitate antibody penetration. This buffer was also used for the dilution of the antisera and the washing steps. Incubation in appropriately diluted primary antisera (see below) for 12–72 h at 4 °C was followed by indirect immunofluorescence staining with fluoresceinisothiocyanate-coupled goat anti-rabbit IgG (FITC–GAR; Sigma, Taufkirchen, Germany) diluted 1:30 in PTX and applied overnight at 4 °C. Preparations were mounted in a glycerol–water mixture (1:1), viewed under a Leitz Orthoplan fluorescence microscope with an Hg epi-illumination (HBO 200W/4) and documented on Kodak T-Max 400 film.

Peroxidase–antiperoxidase (PAP) staining of serial 7 μm thick paraplast sections of ganglia or whole anterior bodies was as described earlier (Dircksen *et al.* 1988), following the essentials of Sternberger (1979). In brief, primary antisera were applied for 1–2 h at room temperature or longer (12–48 h) at 4 °C. Subsequent incubations with the secondary antibody (GAR, diluted 1:30) and the PAP complex (Biogenzia–Lemania, Bochum, Germany; diluted 1:100) followed, both for 1 h at room temperature. The peroxidase reaction was done under visual control in 0.05% 3,3′-diaminobenzidine tetrahydrochloride (Sigma) and 0.015% H_2O_2 buffered with 0.05 M Tris–HCl buffer (containing 0.9% NaCl) at pH 7.6 for 3–6 min.

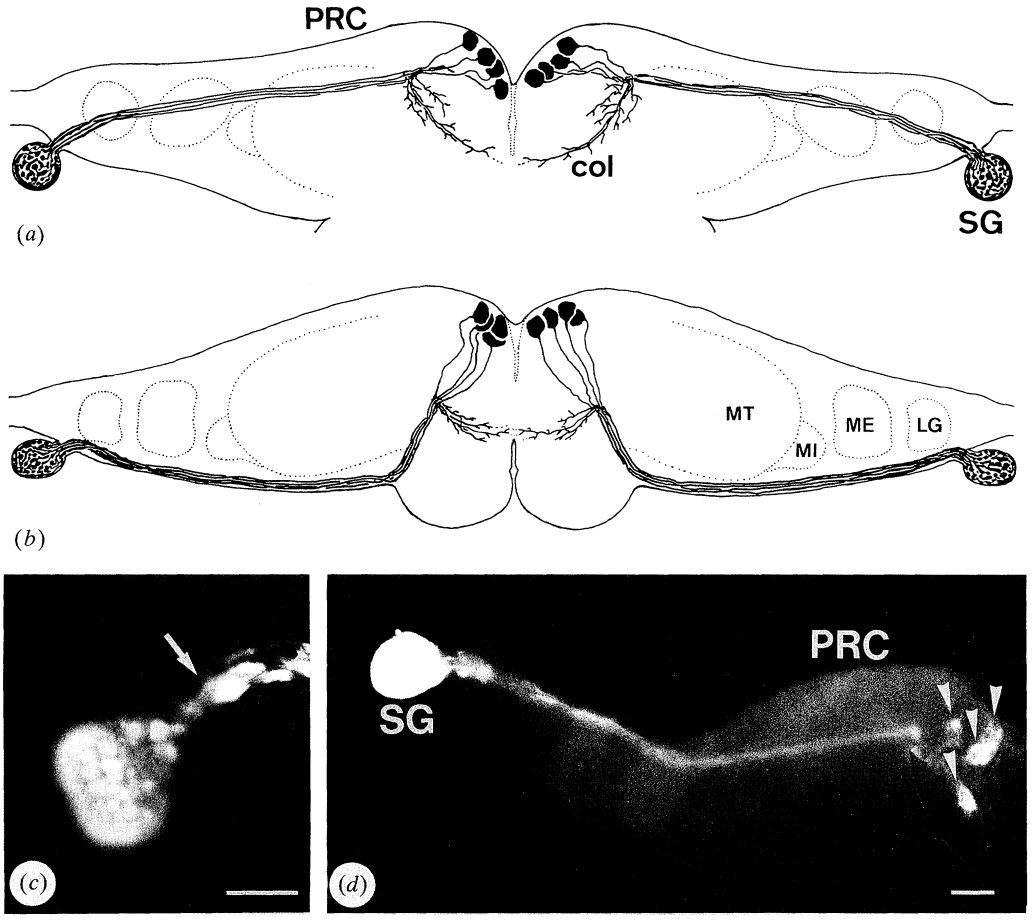

Figure 1. Semidiagrammatic drawing of eight CHH-immunoreactive neurons in a classical neurosecretory pathway of the protocerebrum (PRC) of *Oniscus asellus* as depicted from whole-mount preparations. (*a*) Frontal view. (*b*) Dorsal view. (*c*) Sinus gland (SG) at the posterior lateral margin of the optic lobe; note the bulbous varicosities (arrow) and the abundant terminal axon swellings. (*d*) Perikarya in the isopod medulla terminalis X-organ equivalent and the axon tract to the SG of a hemibrain; note the strong immunoreactivity of the SG in contrast to the faint staining of the tract and the somata. Whole-mount immunofluorescence preparations in (*c*,*d*). Scale bars: 50 μm.

Sections were dehydrated in graded ethanol series and mounted in DePeX (Serva, Heidelberg, Germany). For PAP staining of whole-mount preparations, incubations in GAR (diluted 1:30) and PAP complex (diluted 1:300) lasted overnight at 4 °C. The peroxidase reaction was done for up to 15 min under visual control in the same solution as described above but with 0.0015% H_2O_2. Specimens were examined with use of bright field optics at a Leitz Orthoplan microscope or differential interference contrast at a Zeiss Axioskop and documented on Agfapan 25 film. Leitz optics were used for camera lucida drawings of whole-mount preparations. At least 15 successfully stained preparations out of more than 50 specimens were analysed for each antiserum.

(*c*) *Antisera and specificity tests*

Production and characterization of the polyclonal rabbit antisera used in this study have been described previously in detail elsewhere. Antisera against HPLC-purified crustacean hyperglycemic (CHH) and moult inhibiting hormone (MIH) of *Carcinus maenas* (see Dircksen *et al.* 1988; code anti-CHH 1B1/4, anti-MIH 1TB) and antisera against synthetic pigment dispersing hormone (β-PDH) of *Uca pugilator* (Dircksen *et al.* 1987; code anti-PDH 3B3) were diluted 1:500 for whole mounts and 1:1000–1:5000 for staining of sections. An antiserum against the N-terminal tetrapeptide pyroGlu–Leu–Asn–Pro common to crustacean red pigment concentrating hormone (RPCH) and insect adipokinetic hormone (AKH; code anti-AKH 433 generously supplied by Dr H. Schooneveld, Wageningen, The Netherlands; see Schooneveld *et al.* 1986) was used at the same dilutions. This antiserum has been successfully used in several studies to demonstrate RPCH in crustacean nervous systems (Mangerich *et al.* 1986; Gaus *et al.* 1990; Dircksen 1992).

The specificity of immunostaining was checked by preabsorption of the primary antibodies with their appropriate antigens applied to the final serum dilutions for 24 h at room temperature: 1 μl anti-CHH serum was preabsorbed with 10 μg HPLC-purified *Carcinus* CHH (*ca.* 1.2 nmol), 1 μl anti-PDH serum with 20 μg synthetic *Uca* PDH (*ca.* 10 nmol; generous gift of Drs K.R. Rao and J.P. Riehm, Pensacola, Florida, U.S.A.) and 1 μl anti-AKH/RPCH serum with 100 μg *Locusta* AKH (*ca.* 100 nmol) or the same amount of *Pandalus* RPCH both supplied as synthetic

peptides (Peninsula). All these treatments abolished immunostainings completely.

3. RESULTS

(*a*) *Gross morphology of the central nervous system*

A detailed morphological description of the central nervous system (CNS) of *Oniscus asellus* has been provided earlier by Walker (1935). The abbreviations used in this study refer to the nomenclature of this work unless otherwise stated. Positional information is given with respect to the body axis of the adult isopod.

The brain of *Oniscus asellus* consists of the protocerebrum (PRC), the reduced deutocerebrum (DC) and the large tritocerebrum (TC). The elongate slender lobes of the PRC include the optic lobe with the neuropils lamina ganglionaris, medullae externa, interna and terminalis (ME, MI, MT) typical for malacostracan crustaceans and a pedunculate bulb-like sinus gland protruding posteriorly from the optic lobe (figures 1 and 3). The tritocerebral lobes border the orifice of the oesophagus. The TC directly joins the mouthpart ganglia, namely the mandibular (MD), the first (MX1) and the second maxillary ganglion (MX2). Next to the region of the MD, the entire brain is oriented in a frontal plane almost at a right angle to the VNC. The subsequent VNC ganglia comprise eight thoracic (T1–T8) and six fused abdominal ganglia (AG = A1–A6 neuromers; compare with figure 3). The thoracic ganglia are uniform, except for the first and the last ones. The first is fused with the mouthpart neuromers to form the so-called maxillipedal ganglion (MXP = T1), which is contained in the head capsule. The thoracic ganglia T2–T7 are free, whereas the T8 is fused to a compact mass with the AG.

(*b*) *CHH-immunoreactive neurons*

CHH immunoreactivity in the CNS of *Oniscus asellus* is confined to a system of four bilaterally symmetrical neurons in the PRC as shown in the composite diagram of figure 1*a*,*b*. The polygonal cell bodies (17–23 μm in diameter) are localized in an anterior median position of the PRC. Their cytoplasm shows a granulated appearance in whole-mount immunofluorescence preparations (figure 1*d*). The primary neurites project to the central PRC neuropil. At this point collaterals are given off in a medial ventral direction, and the axons fasciculate to form a prominent tract. Collaterals form dendritic branchings which may reach the contralateral hemisphere. This could not clearly be established, since the immunostaining tended to fade in the higher order branches. The axonal tract (figure 1*d*) leaves the median neuropil and turns in a lateral direction. Along the posterior surface of the optic lobe, it runs distally and enters the sinus gland (SG) where numerous large axonal swellings and terminals are detectable (figure 1*a*,*b*,*c*). The SG is always more intensely stained than the somata. The staining intensity of the axonal tract increases towards the periphery, and bulbous varicosities and axon swellings can be observed on its distal course (figure 1*c*,*d*).

(*c*) *MIH immunoreactivity*

Neither in whole-mount preparations nor in paraplast sections have immunopositive structures been detected with the applied anti-Cam-MIH antiserum.

(*d*) *PDH-immunoreactive neurons*

PDH-immunoreactive neurons are confined to the PRC as is shown in a semi-diagrammatic composite drawing (figure 2*a*) and in a reconstruction of the entire system in one protocerebral hemisphere (figure 2*b*). In total 16–18 perikarya are found in the PRC, whereas no somata could be detected in other ganglia of the CNS. Eight bilaterally symmetrical neurons occur consistently. They have been divided into three distinct PDH-immunoreactive groups (PGR1, PGR2, PGR3) that can be distinguished with regard to their staining intensities, the size of their somata, and their projection patterns.

(i) *PGR1 and PGR2 neurons*

The three perikarya of the PGR1 neurons and the two perikarya of the PGR2 neurons are localized in the anterior median cell layer of each PRC hemisphere (figure 2*b*,*f*). Their positions vary to some extent in different specimens, but usually occur in more dorsal positions than those of the CHH-immunoreactive cells. PGR1 neurons show slightly larger (15–17 μm in diameter) and more irregularly shaped perikarya, exhibit weaker immunostaining and bear larger axons than the PGR2 neurons (12–15 μm in diameter). Axonal processes of both cell groups run to an elaborate star-shaped median plexus. At this point, the further course of the axons was difficult to trace with certainty. However, since three distinct thick axons emerge from the median plexus and enter the SG, they obviously arise from the three large PGR1 perikarya (figure 2*b*,*e*). This became particularly clear after the analysis of a single anomalous specimen, in which the PGR1 neurons of one hemisphere lacked one perikaryon and consequently showed merely two axons running into the SG. In the MT neuropil, the PGR1 axons join the axonal tract of the CHH-immunoreactive neurons and give rise to numerous small terminals in the SG bulb (figure 2*d*). PGR2 neurons are probably the origin of two faintly stained longitudinal fibres, one descending through the mouthpart ganglia down to the MXP, the other descending through the entire VNC down to the AG (figure 2*a*,*c*). On their way, both fibres give rise to collaterals in segmental neuropils (figure 2*c*). However, it was impossible to trace their suspected connection back to the PGR2 neurons (figure 2*a*, arrowheads) with certainty within the deutocerebrum and the tritocerebrum.

(ii) *PGR3 neurons*

This group of neurons consists of the three most intensely stained perikarya (17–22 μm in diameter) localized within the optic lobe between the ME and the MI–MT complex (figure 2*b*,*e*,*f*). They give rise to T-

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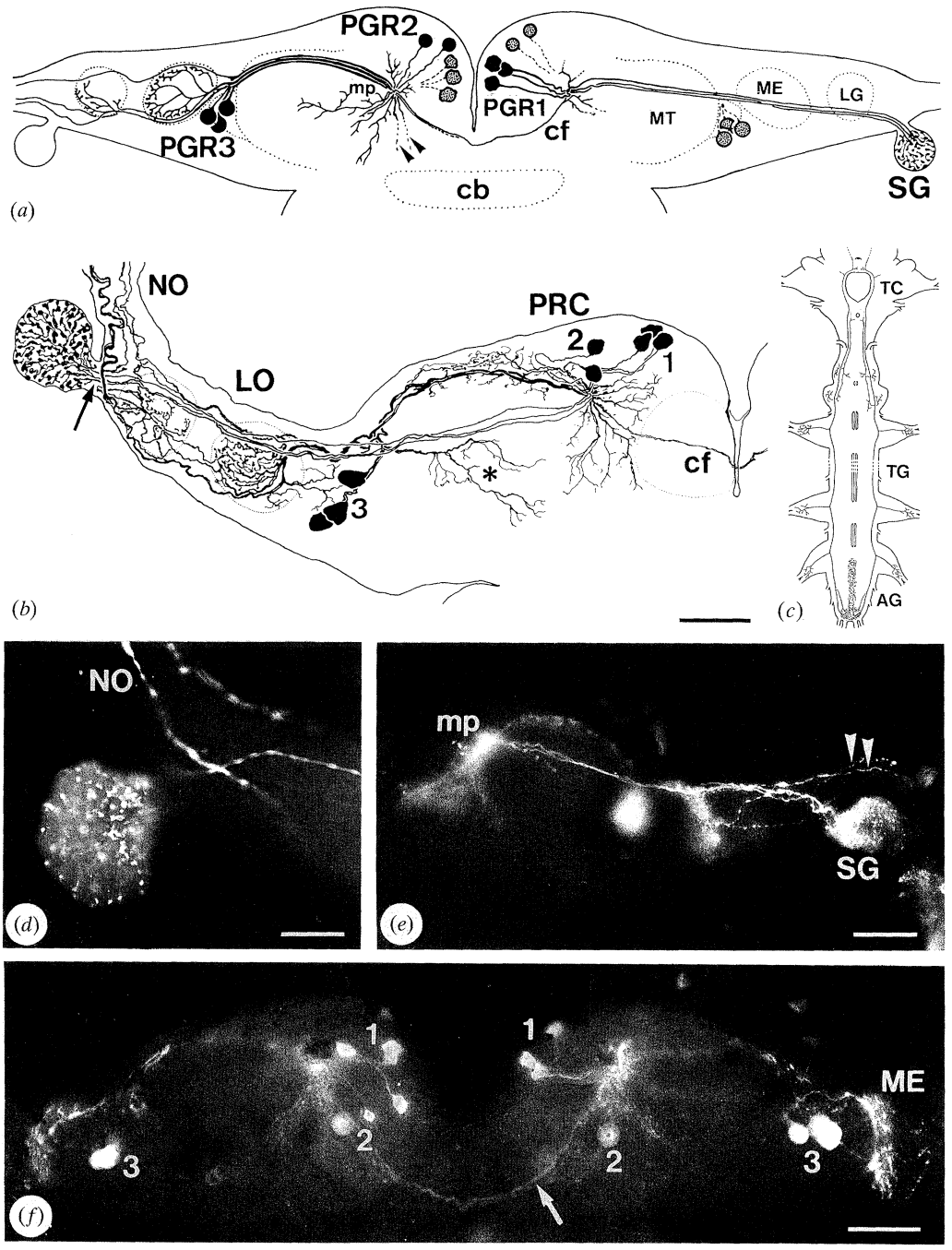

Figure 2. PDH-immunoreactive neurons in the CNS of *Oniscus asellus*. (*a*) Composite diagram of the brain (frontal view) showing three identified groups of neurons (PGR1–3) and their projection patterns as depicted from whole-mount preparations. PGR1 neurons are shown in the right PRC hemisphere, PGR2 and PGR3 neurons in the left. Arrowheads indicate the proposed origin of the fibres shown in (*c*) (cf, commissural fibre; mp, median plexus). (*b*) Camera lucida drawing of all neurons of a left PRC and optic lobe (LO; posterior view; whole-mount preparation). Note the three PGR1 axons entering the SG (arrow), the central arborization of proximal PGR3 axons in the medulla terminalis (asterisk) and the commissural fibre (cf). (*c*) Schematic drawing of two descending fibres in the ventral nerve cord (VNC) which apparently belong to the PGR2 neurons. Note the collateral branchings. No immunopositive perikarya occur in the VNC. (*d*) Sinus gland showing small and sparsely distributed immunopositive axon terminals. Note two PGR3 axons passing through the optic nerve (NO). (*e*) Projections and fibre tracts within a left PRC (frontal view). Note the axon bundle running directly from the mp to the sinus gland (SG) and PGR3 fibres extending into the optic nerve (arrowheads). (*f*) Neuron groups in the median PRC. Note the commissural fibres (arrow), the median plexus (mp), and the lateral plexus of the medulla externa (ME). Whole-mount immunofluorescence preparations in (*d*)–(*f*). Scale bars: (*b*,*d*) 50 μm; (*e*,*f*) 100 μm.

shaped axons with distal and proximal branches. The latter branches form a strongly stained axon bundle (figure 2*b*,*f*). Several fine collaterals emerge from this bundle in the central MT neuropil (figure 2*b*, asterisk). The distal branches split into single fibres that partly supply a diffuse plexus within the medulla externa and, to a lesser extent, within the lamina ganglionaris. Three to five distinct fibres of these branches extend

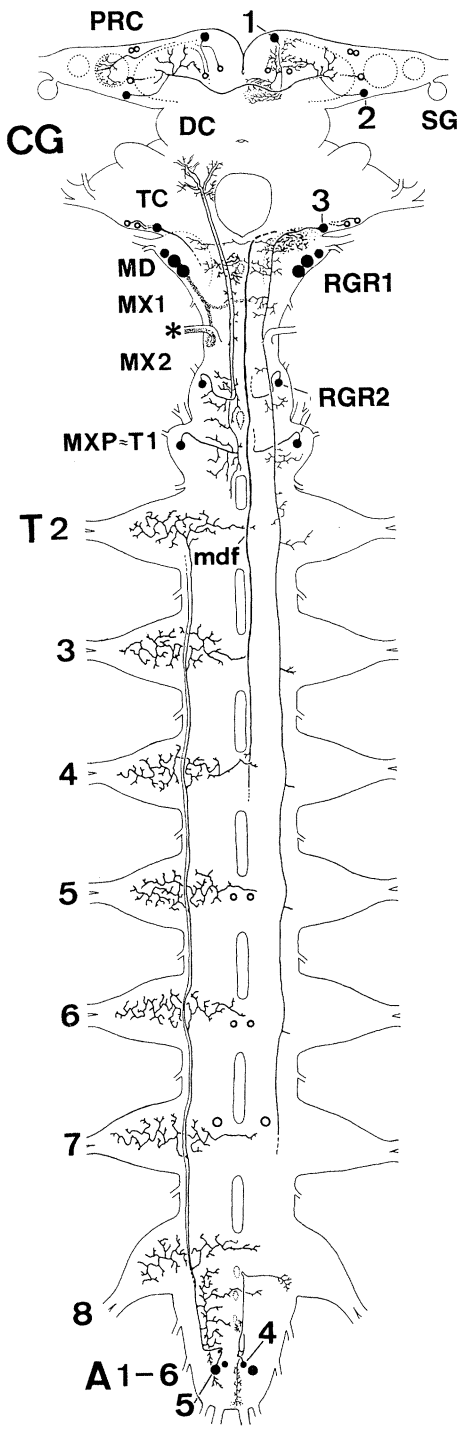

Figure 3. Schematic drawing of the entire CNS of *Oniscus asellus* showing identified RPCH-immunoreactive groups of neurons (RGR1, RGR2), individual neurons types (1–5), unidentified fibres (mdf, median dorsal fibre) and plexuses. Open circles indicate weakly stained perikarya (e.g. RGR1). Complete sketches of each individual neuron type are only drawn for a hemiganglion. Asterisk indicates the thick dorsal nerve of MX2 containing peripheral projections of the RGR1 neurons.

into the optic nerve (NO; figure 2*b*,*d*). These fibres can be followed to the basis of the ommatidial cells of the compound eye (figure 2*e*, arrowheads).

All three groups of PDH-immunoreactive neurons contribute to the median plexus in the central optic neuropil of a PRC hemisphere. This plexus gives rise to contralaterally projecting fibres (cf) that connect the left and the right hemisphere and are part of a commissure dorsal to the central body (cb; figure 2*a*,*b*). The axon of one weakly immunopositive neuron in the median PRC dorsal to the cell bodies of PGR1 and PGR2 could also be traced to this plexus, but was not consistently observed.

(*e*) *RPCH-immunoreactive neurons*

A total number of 20 strongly stained RPCH-immunoreactive neurons in the entire CNS occurs regularly in all preparations. Their distribution is summarized in the composite diagram of figure 3. Additional weakly stained perikarya (about 20 in total) occur in the brain and the posterior thoracic ganglia. They were not consistently observed and are outlined as open circles in figure 3. Two sets of neurons have been grouped (RPCH-immunoreactive groups RGR1 and RGR2), because the pathways of subtypes of their neurons showed striking similarities. Another five individual neuron types (1–5) have been reconstructed in more detail. Furthermore, a few immunopositive fibres and plexuses of unknown origin were observed.

(i) *RGR1 neurons*

Two adjacent subtypes of RGR1 neurons occur in lateral positions within a cell cluster of the mandibular ganglia, the 'cellulae mandibulares' (CMD of Walker 1935). The first subtype, represented by two cells on each side, is characterized by a large perikaryon (22–30 μm in diameter) and the granular appearance of its faintly stained cytoplasm in whole-mount preparations (figure 4*b*). A weakly staining axonal tract originating from these neurons runs in caudal direction and gives off contralaterally arborizing collaterals in the first maxillary ganglion (MX1; figure 4*d*,*e*). The axons continue via the thick dorsal segmental nerve of the second maxillary ganglion (according to Walker (1935) MX2; figures 3, 4*b*) to the lateral cephalic nerve plexus (LCNP), where they form varicosities and putative terminals at the surface of this neurohaemal organ in close association to the Y-organ (figure 5*a*,*b*). The single perikaryon on each side of the second subtype of the RGR1 neurons is medium-sized (19–24 μm in diameter) and densely stained and shows a coarsely granulated cytoplasm in sections. Its axon could not be visualized completely but probably joins the peripherally directed fibre bundle of the first RGR1 subtype (figure 4*b*).

(ii) *RGR2-neurons*

This group comprises two similar ascending neurons in the MX2 and MXP/T1 neuromers; one soma is localized in the ventral part of the second maxillary ganglion (MX2), the other in a dorsal position of the maxillipedal ganglion (MXP). Both measure about 12 μm in diameter and exhibit similar ipsilateral projection patterns (figures 3; 4*c*): Their axons run transversely to the midline, form characteristic median

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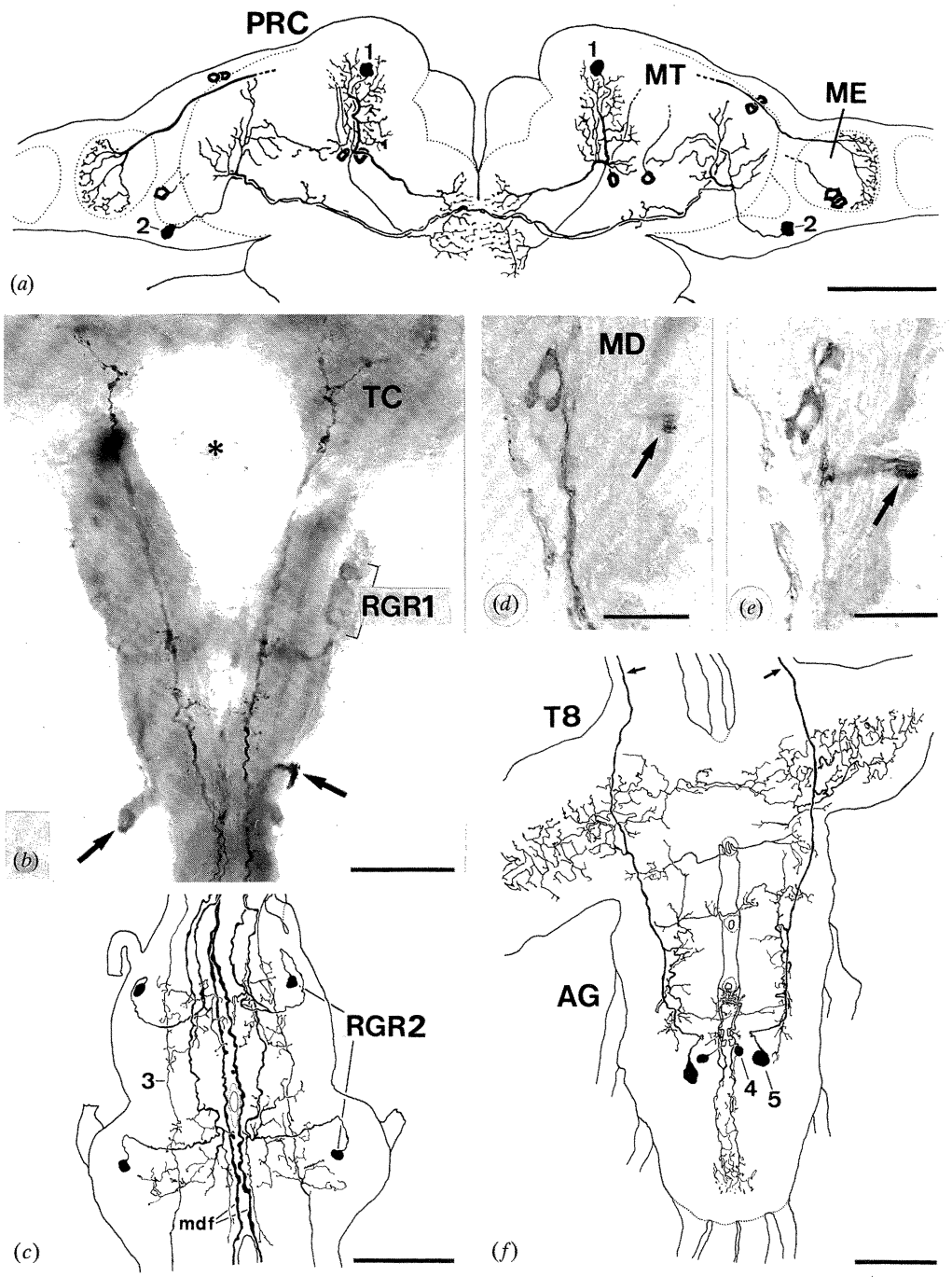

Figure 4. RPCH-immunoreactive neurons in the CNS of *Oniscus asellus*. (*a*) Pathways of type 1 and type 2 neurons and occasionally occurring weakly stained perikarya (unfilled perikarya) in the PRC (frontal view; camera lucida drawing of a whole-mount preparation). (*b*) Weakly stained perikarya of RGR1 neurons projecting into the thick dorsal nerves of MX2 (arrows) leading to the lateral cephalic nerve plexus (not shown), and ascending pathways of RGR2 axons to the tritocerebrum (TC; dorsal view of a PAP-stained whole-mount preparation; compare with (*c*)). Asterisk indicates the orifice of the oesophagus. (*c*) Camera lucida drawing of the RGR2 neurons in the MX2 and MXP following caudally upon (*b*) (same whole mount as in (*b*)). Note the ipsilateral projection patterns. (*d*,*e*) Perikarya of RGR1 neurons and their processes in consecutive PAP-stained longitudinal sections through the mandibular ganglion (MD). Arrows point to the central projections. (*f*) Median type 4 and ascending type 5 neurons (arrows) of the fused abdominal ganglia complex (AG; camera lucida drawing of a PAP-stained whole mount). Note that the small type 4 neurons are restricted to the abdominal ganglia. Scale bars: (*a*,*b*,*c*,*f*) 100 μm; (*d*,*e*) 25 μm.

arborizations and ascend into the TC; on their way they give off side branches in each ganglion (figures 3, 4*b*,*c*). No contralateral extensions of these collaterals are detectable. In the central neuropil of the TC next to the orifice of the oesophagus, both RGR2 axons terminate in a star-shaped arborization (figure 4*b*).

(iii) *Neuron of type 1*

The soma of the type 1 neuron (about 10–12 μm in diameter) is localized in the superior lateral cells (CSL of Walker (1935) at the dorsal surface of the median PRC. The primary neurite projects ventrally into the central optic neuropil where it ramifies into three

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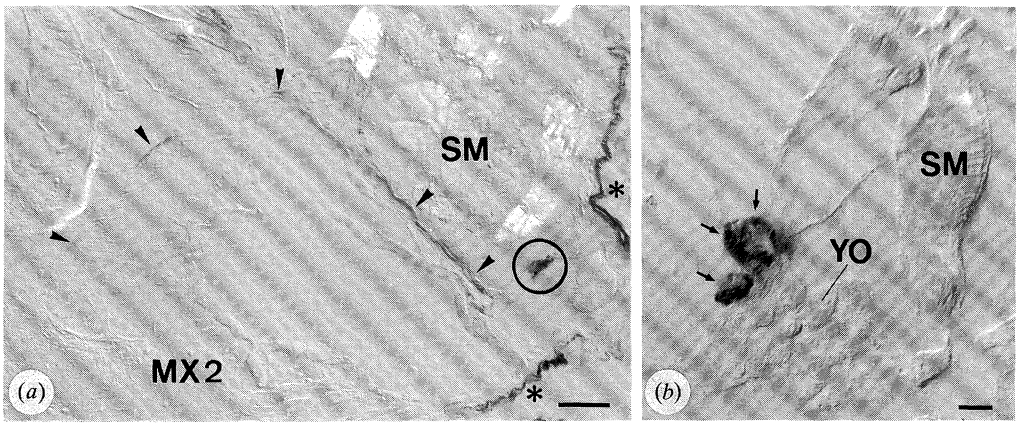

Figure 5. Peripheral RPCH-immunoreactive structures in the right dorsal nerve of the MX2 of *Oniscus asellus* consist of axons ((*a*), arrowheads) of RGR1 neurons supplying the lateral cephalic nerve plexus (LCNP; encircled) in the vicinity of the ventral lateral integument (asterisks) and somatic muscles (SM). Some of these fibres pass the Y-organ (YO), which is closely attached to the LCNP (*b*), or form endings next to the surface of this nerve plexus (arrows). PAP-stained paraplast cross sections through the whole head. Scale bars: (*a*) 50 μm; (*b*) 10 μm.

distinct processes (figure 4*a*). Two of these proceed in a ventral and median direction, and finally arborize in neuropils dorsal and ventral to the immunonegative central body. Contralateral projections are lacking. The third branch runs recurrently parallel to the primary neurite and forms an elaborate terminal arborization in a neuropil area posterior to the soma (figures 3, 4*a*).

(iv) *Neuron of type 2*

The contralaterally projecting type 2 neuron occurs ventral and proximal to the ME of the optic lobe (figure 4*a*). Its perikaryon (about 12 μm in diameter) sends a fine primary neurite into the central neuropil of the MT, where it gives off several collaterals (figures 3, 4*a*). The axon runs counterparallel to the axon of its contralateral twin towards the contralateral hemisphere. Upon joining the first branching point of its contralateral twin, it forms a terminal arborization. Thus, the projection patterns of the neurons of both sides form a mirror image.

(v) *Neuron of type 3*

The strongly immunofluorescent perikaryon of the type 3 neuron (about 10–14 μm in diameter) is localized in the posterior cell cluster of the tritocerebrum (CTrcI of Walker 1935). The axon descends through the mouthpart ganglia and at least the first seven thoracic ganglia. It could not be traced to its final terminations because the staining intensity decreased in the distal parts. This neuron forms a conspicuous arborization in the mandibular ganglion and gives off single collateral branches in all subsequently following ganglia (figure 3).

(vi) *Neuron of type 4*

The polygonal soma of the faintly immunopositive type 4 neuron measures 10–12 μm in diameter and lies in the centre of the ventral cell monolayer (cellulae abdominales) of the posterior AG complex between the A3 and A4 neuromers (figure 4*f*). The intricate axonal pathway and branching pattern of this neuron are mainly restricted to the median abdominal neuropils (figures 3, 4*f*). The axon climbs up to the dorsal ganglionic surface, where it gives off two ascending and one descending branches. The larger of the ascending branches forms elaborate arborizations in the median neuropil of the A3 neuromer. The smaller one proceeds to the A1 neuromer, turns laterally and forms a terminal arborization in the lateral neuropil of this neuromer but also contributes a collateral to the A3 neuromer. The descending branch forms median plexuses within the A4 down to the A6 neuromers (figure 4*f*).

(vii) *Neuron of type 5*

The strongly immunopositive type 5 neuron is localized close to the type 4 neuron but in a more lateral and caudal position. Its large soma (15–22 μm in diameter; figure 4*f*) gives rise to an axon that runs from the ventral to the dorsal ganglionic surface, similar to that of the type 4 neuron, but ascends after turning in lateral direction and forms terminal arborizations in T2 (figure 3). At least two collaterals branching off the main axon form a longitudinal plexus that extends from the A4 up to the A1 neuromers. In each ganglion from the T7 to the T3, one collateral gives rise to a conspicuous ipsilateral ventral arborization and some small contralateral branches (figure 4*f*).

(viii) *Other, less well characterized RPCH-immunoreactive neuronal structures*

An immunopositive fibre running next to the dorsal surface of the PRC into the distal optic lobe supplies a weakly stained fibre plexus in the outer neuropil layer of the medulla externa. Its origin could not definitely be related to one of the above mentioned weakly

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stained perikarya of the PRC. All other distal parts of the optic lobe, the lamina ganglionaris, the optic nerve and the SG are immunonegative.

Median to the axons of RGR2 neurons a longitudinal median dorsal fibre (mdf, figure 4*c*) occurs. This strongly immunopositive varicose fibre shows no branches. It can be followed from the mandibular to the fourth thoracic ganglion. Further tracing was impossible because of faint immunostaining, and its origin could not be established.

In the thoracic ganglia, a fine longitudinal fibre (figure 3), not detectable in all preparations, was found to run parallel to the axon of the type 5 neuron, but its origin and termination sites remain unclear.

4. DISCUSSION

With the aid of cross-reacting antisera against the well established decapod neurohormones CHH, PDH and RPCH we have demonstrated three distinct neurosecretory pathways transporting neurosecretory products to neurohaemal organs, the sinus gland (SG) or the lateral cephalic nerve plexus (LCNP) of *Oniscus asellus*. The principal organization of the identified isopod CHH-immunoreactive and PDH-immunoreactive neurons resembles to a certain extent the decapod patterns, while the isopod RPCH-immunoreactive neurons are obviously different. This is the first immunocytochemical mapping and almost complete tracing of neurons putatively containing neuropeptides related to the decapod PDHs and RPCH in an isopod species. Apart from neurosecretory neurons, our results demonstrate newly discovered PDH-immunoreactive and RPCH-immunoreactive putative interneurons.

(*a*) *The isopod 'X-organ–sinus gland system'*

The median protocerebrum (PRC) of isopods has long been known to contain classical neurohormones regulating blood sugar level, moult, growth, reproduction and pigmentation of these animals (Mocquard *et al.* 1971; Juchault & Kouigan 1975; Martin 1988). Chiang & Steel (1985*a*,*b*, 1986), who studied in detail the SG-innervating neurons in *Oniscus asellus*, have found that both β-cells in the anterior median PRC and B-cells in the central PRC project into the SG bulb. The authors have merely discussed MIH as possible hormone content but have ignored a previously characterized isopod CHH, the Pod-CHH (Martin *et al.* 1984*a*). Criteria such as soma position, projection patterns and the cytological changes of β-cells during the moult cycle led the authors to assume that the B-cells *and* the β-cells together comprise the 'isopod equivalent of the decapod X-organ', whereas a third projection to the SG bulb originates from small γ-cells located in the optic lobe. This view has been in good agreement with earlier suggestions based upon similar morphological criteria and conclusions about the physiological actions of the proposed hormone contents (Legrand *et al.* 1968; Juchault & Kouigan 1975). Are these criteria sufficient for considering these isopod cell clusters homologous to the decapod X-organ cells, or can our immunocytochemical identification of SG-innervating cells according to their putative contents of 'decapod-like' hormones help to solve the homology problem at the level of individually identified cells?

(i) *CHH and MIH immunoreactivity*

The architecture of the CHH-immunoreactive neurons of *Oniscus asellus* and of other oniscoid species such as *Porcellio scaber* and *Ligia oceanica* (T. Nussbaum & H. Dircksen, unpublished observations) matches almost completely the system of two CHH-immunoreactive β-type neurons demonstrated for the terrestrial isopod, *Porcellio dilatatus*, by Martin *et al.* (1984*b*). However, in *Oniscus asellus*, *Porcellio scaber* and *Ligia oceanica*, four immunopositive neurons occur in each hemisphere. In *Oniscus asellus*, the CHH-immunoreactive neurons are obviously identical to the β-cells described by Chiang & Steel (1984, 1985*a*) and may represent a subset of the six paraldehyde fuchsin-positive β-cells per hemisphere described by Messner (1966). According to Martin (1981, 1988), the β-cells in *Porcellio dilatatus* and *Ligia oceanica* can be further subdivided into three β_1-cells and two β_2-cells per hemibrain. In these species they are distinguishable because of slight morphological differences and granule types. In *Oniscus asellus*, a similar heterogeneity has been inferred by Chiang & Steel (1986) based upon the analysis of action potentials. By use of an antiserum against crude *Carcinus* SG extracts (Jaros & Keller 1979), Martin (1988) found immunoreactive granules in β_1-perikarya and in the most abundant type I axon terminals of the SG in *Porcellio dilatatus* and considered them to be CHH-immunopositive structures. We have used a different, highly specific antiserum against purified Cam-CHH, and may thus be dealing with four CHH-immunoreactive β_1-neurons but perhaps another two β_2-neurons with a hitherto unknown neurosecretory product in the closely related species *Oniscus asellus*. The latter or even both β-cell subtypes may contain a vitellogenesis inhibiting hormone (VIH) as proposed earlier by Martin (1972) and Demassieux & Balesdent (1977). Indeed, immunoreactivity to a decapod VIH antiserum has been shown to be mainly associated with type I granules of SG terminals in *Porcellio dilatatus* (Meusy *et al.* 1987) which originate from the β-cell cluster (Martin 1981, 1982). The results concerning this pathway resemble those obtained later for the CHH-immunoreactive and VIH-immunoreactive neurons in the X-organ–SG system of *Homarus americanus* (Kallen & Meusy 1989).

Another important structural detail of the CHH-immunoreactive neurons in *Oniscus asellus* are the collateral branchings and preterminal swellings within the axonal tract which are similar to those described for the β-cells by Chiang & Steel (1985*a*). These structures have possibly been overlooked in the study of Martin *et al.* (1984*b*). However, dendritic collaterals of CHH-immunoreactive neurons commonly occur in decapod crustaceans and have been considered as areas of interneuronal modulatory input into this classical neurosecretory system (Gorgels-Kallen 1985; Dircksen *et al.* 1988). In astacurans, these collaterals

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are known to receive serotoninergic synaptic input (Van Herp & Kallen 1991). This may account for stimulation of CHH release and hyperglycemia, a serotonin effect that has been observed in different decapod species (Keller & Beyer 1967; Strolenberg & Van Herp 1977). Martin (1978) showed similar biological effects of serotonin injections for the isopod *Porcellio dilatatus*. These effects coincided with increased exocytotic activities in type I and type II terminals of the SG of this species. It would be interesting to investigate whether β-cell collaterals, in addition, receive visual inputs as is known from decapod X-organ–SG cells (Glantz *et al.* 1983). Such inputs would explain the daylength-controlled SG hormone release suggested by Steel (1980) as being responsible for the induction of breeding in female *Oniscus asellus*.

Because the X-organ–SG cells in the decapod *Carcinus maenas* had clearly been differentiated by the anti-CHH and anti-MIH antisera (Dircksen *et al.* 1988; Dircksen 1992), we expected a similar architecture of CHH-immunoreactive and MIH-immunoreactive cells in *Oniscus asellus*. However, MIH immunoreactivity does not occur in *Oniscus asellus*, which leaves the question whether a MIH-like molecule exists in isopods. Since Cam-MIH seems to exhibit pronounced species or group specificity (Webster 1986) and MIH immunoreactivity has thus far only been detectable in brachyurans with our anti-Cam-MIH antiserum (Dircksen *et al.* 1988), the existence of a cross-reactive Cam-MIH-related substance in isopods seems unlikely. On the other hand, it seems worth investigating whether the isopod CHH molecules (Martin *et al.* 1984*a*, 1993) display moult inhibiting activity as has been established for some molecular forms of CHHs in *Carcinus maenas* and *Homarus americanus* (Webster & Keller 1987; Chang *et al.* 1990). If this were so in isopods, it would explain the close relation between the moult cycle and the secretory activity of β-cells in different isopods observed by several authors (Gabe 1952; Messner 1966; Martin 1972; Martin *et al.* 1983; Chiang & Steel 1985*a*, 1987). To date, it is unclear whether the current concept of moult control in decapods is adequate for an explanation of the complex biphasic moulting process of isopods, since experimental evidence has been provided for both moult inhibiting and moult accelerating principles (Carlisle 1956; Mocquard *et al.* 1971; Charmantier 1978; Martin *et al.* 1979, 1980, 1983). However, all available data strongly suggest that the CHH-immunoreactive neurosecretory cells of the isopod β-cell–SG system and the decapod X-organ–SG system are true homologues and may play a dual role in the control of haemolymph glucose levels and moulting in both malacostracan groups.

(ii) *PDH-immunoreactive neurons innervating the sinus gland*

In *Oniscus asellus*, as in the decapod species *Carcinus maenas* and *Orconectes limosus* (Mangerich *et al.* 1987), there are three PDH-immunoreactive axons that innervate the SG. However, the origins of these axons may be different in both malacostracan suborders though detected by use of the same anti-PDH serum that is highly specific for β-PDH (Dircksen *et al.* 1987; Bonomelli *et al.* 1988; Löhr *et al.* 1993). In the decapod species, these axons do not arise from X-organ cells but from a conspicuous immunopositive fibre tract apparently originating mainly from primary neurites of perikarya between the medullae interna and terminalis of the eyestalk ganglia. This position is similar to the SG-innervating γ-cells of *Oniscus asellus* described by Chiang & Steel (1985*a*,*b*). However, the γ-cells are PDH-immunonegative and, in contrast to the decapod species, the three PDH-immunoreactive SG-innervating PGR1 neurons in *Oniscus asellus* originate from the superior median PRC. With regard to their size, their position dorsal to the CHH-immunoreactive β-cells, and their projection patterns along the CHH-immunoreactive tract to the SG, they are most likely identical with the SG-innervating neurosecretory B-cells (Chiang & Steel 1985*a*,*b*). In a position similar to that of the PGR1 neurons in *Oniscus asellus*, three distinct PDH-immunoreactive perikarya have been described in the anterior median cell cluster of the decapod brain (Mangerich & Keller 1988). These neurons probably give rise to fibres that run through the optic nerve and join the above-mentioned MT tract in the eyestalk. However, there was no evidence for a possible origin of the three PDH-immunoreactive SG-innervating axons in these decapod brain cells. Thus, the morphological data seem to argue against a putative homology of the PDH-immunoreactive SG-innervating neurons in decapods and isopods. Hence, it seems unlikely that the SG-innervating B-cells of *Oniscus asellus* are a homologous part of the 'isopod equivalent of the decapod X-organ' according to the hypothesis of Chiang & Steel (1985*a*).

Although evidence has been provided for the existence of PDH bioactivity in the PRC and in the SG of different marine and terrestrial isopod species (see Castrucci & Mendes 1975), including *Oniscus* species (Stahl 1938*a*,*b*), the hormonal function of PDH-immunoreactive substances in terrestrial isopod species remains unclear. Since these species seem to lack functional chromatophores, other hitherto unknown targets must be postulated for a PDH released from the SG. In decapods, evidence has been provided for an antagonistic control of the mandibular organ by a PDH and RPCH (Landau *et al.* 1989). These endocrine organs are known to release methyl farnesoates as hormonal substances that control a variety of reproductive functions (Laufer *et al.* 1993). However, since an isopod equivalent of the mandibular organs has not yet been described, it seems premature to assume a similar structure as a possible target of chromatophorotropins in isopods.

(*b*) *The RPCH-immunoreactive neurosecretory pathway to the lateral cephalic nerve plexus*

The existence of an RPCH-like substance in terrestrial isopods has hitherto been demonstrated only in a few biotests: brain extracts of *Armadillidium vulgare* led to the concentration of black pigment in three marine isopod species (Okay 1945). Extracts of the VNC of *Trachelipus rathkei* show a concentrating effect on erythrophores of a *Cambarus* species (McWhinnie &

Sweeney 1955). Since biochemical investigations on the characterization of chromatophorotropic molecules in isopods are lacking, it cannot be excluded that the antiserum used in this study has identified neurons containing molecules closely related but different from 'authentic' RPCH. Schooneveld *et al.* (1987) provided, to our knowledge, the only immunocytochemical evidence for peptides related to the AKH–RPCH peptide family (Gäde 1992) in an isopod by mapping some immunoreactive somata in the brain of the sowbug *Porcellio scaber*. No immunoreactivity was found in the SG or in the VNC. The authors, however, used a different antiserum produced against the carboxyl terminus of a [Tyr^1] analogue of the locust decapeptide AKHI (Schooneveld *et al.* 1983, 1987). Surprisingly, these authors did not find any immunoreactivity with the antiserum used in the present study, although it obviously recognizes the common N-terminal tetrapeptide of AKHI and RPCH equally well, as indicated by our preabsorption controls. Furthermore, most of the cells detected with the anti-AKH serum were in positions different from those described here for *Oniscus asellus*. Thus, these authors probably detected an antigen different from the crustacean octapeptide RPCH, the primary structure of which seems to be conserved in all crustacean species hitherto investigated (Gaus *et al.* 1990).

Since the SG of *Oniscus asellus* is devoid of RPCH immunoreactivity, it is obvious that a neurosecretory RPCH-immunoreactive X-organ–SG system comparable with that found in decapod species (Bellon-Humbert *et al.* 1986; Mangerich *et al.* 1986) is lacking in isopods. This result further supports our view that the term 'isopod X-organ equivalent' is probably only applicable to the CHH-immunoreactive (and VIH-immunoreactive) β-cells. The only RPCH-immunoreactive cells in *Oniscus asellus* projecting into a neurohaemal organ occur in the VNC. The RGR1 neurons of the mandibular ganglion are part of a newly discovered putative neurosecretory pathway to the neurohaemal lateral cephalic nerve plexus (LCNP). A comparable pathway has not been described for any decapod species, but it may be similar to that assumed for suboesophageal neurosecretory neurons in *Sphaeroma serratum* (Chaigneau 1966; Chataigner *et al.* 1978; Maissiat *et al.* 1979) and in *Ligia oceanica* (Martin *et al.* 1983).

The LCNP is a complex anastomosing lateral nervous system unique to isopods. In different isopods, the so-called dorsal nerves of the MX1 and MX2 (Walker 1935) comprise only part of a multiple intersegmental nerve supply to the LCNP (Delaleu 1970) but only that of the MX2 contains RPCH-immunoreactive axons. Because of its close association with the Y-organ as described already in the earliest papers (Silén 1954; Messner 1963, 1966; Besse & Legrand 1964), Martin *et al.* (1983) have discussed in detail a possible involvement of the LCNP in moult control and assumed the existence of a moult accelerating hormone in this neurohaemal area. Whether RPCH-like substances released from the LCNP have to be considered in this respect or regarding the control of other endocrine targets (see above) remains to be investigated, since chromatophorotropic activity of a RPCH seems unnecessary in terrestrial isopods.

(*c*) *Newly discovered putative peptidergic interneurons*

In decapods, the majority of PDH-immunoreactive and RPCH-immunoreactive neurons in the eyestalk and the CNS do not project to neurohaemal organs and seem to comprise merely interneurons (Mangerich *et al.* 1986, 1987). The interneurons of *Oniscus asellus* occur in smaller numbers compared with those of decapods, but some of them show similarities with regard to positions and projection patterns.

(i) *PDH-immunoreactive interneurons*

The perikarya of the three PDH-immunoreactive cell groups are exclusively, and the fibres and plexuses are mainly localized in the protocerebrum. Only two different descending fibres supply neuropils in the suboesophageal ganglia or the entire VNC. In decapods, locally arborizing neurons seem to be restricted to the mouthpart ganglia, to the posterior parts of the brain (DC, TC) and to the lamina ganglionaris (Mangerich *et al.* 1987). In *Oniscus asellus*, no neurons originate in these ganglia. However, similarities with regard to the principal organization of strongly staining neurons between the MI and ME in decapods and the isopod (PGR3) are obvious. The PGR3 neurons do not belong to the SG-innervating γ-cell cluster. Their T-shaped axons supply distal and proximal optic ganglia. In addition, only in the isopod do the distal branches reach the bases of the ommatidia. The function of these unique distal projections in isopods is unclear. In the decapod species, the proximal branches of similar neuron types give rise to the MT tract and may innervate optic neuropils in the brain (Mangerich *et al.* 1987; Mangerich & Keller 1988) as do the proximal branches of the isopod neuron.

In both malacostracan suborders, an additional cluster of neurons occurs in the median rostral protocerebrum. Since the decapod neurons have not yet been reconstructed, we can only assume that the comparable PGR2 neurons in *Oniscus asellus* most likely give rise to the descending fibres in the VNC. For these interneurons spanning large volumes of neuropil a neuromodulatory role cannot be excluded as has been suggested earlier for putative descending interneurons in the brain of decapods (Mangerich & Keller 1988).

Some contralateral projections of PDH-immunoreactive neurons to the median plexus were consistently observed, which suggests that some of these neurons may be electrically coupled. This phenomenon has been previously described for SG-innervating neurons, probably the B-cells, that exhibit this kind of contralateral projections in *Oniscus asellus* (Chiang & Steel 1985*c*, 1987). It has been known for a long time that diurnal rhythms in the activity of chromatophores and retinal pigments occur in decapods (Welsh 1930; Aréchiga & Mena 1975; Rao 1985) and in isopod genera like *Idotea* and *Ligia* (Menke 1911; Piéron 1914, Kleinholz 1937). Both PDH and RPCH are obviously involved in the regulation of these and other circadian

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rhythms (Aréchiga & Mena 1975; Larimer & Smith 1980; Thurman 1988). Interestingly, PDH-immunoreactive neurons in several insects occur in positions similar to those of the isopod PGR3 neurons. Apart from projections to the lamina, these insect neurons show even more extended central and contralateral projections (Homberg *et al.* 1991; Nässel *et al.* 1991, 1993). In orthopteran species, these interneurons obviously participate in the control of circadian locomotory rhythms (Homberg *et al.* 1991; Stengl & Homberg 1992, 1994).

(ii) *RPCH-immunoreactive interneurons*

For the isopod RPCH-immunoreactive interneurons, possible structural homologies to those of decapods are difficult to assess, since the decapod RPCH-immunoreactive putative interneurons have not been reconstructed. All complex neurons (except for the type 3 neuron) in the VNC of *Oniscus asellus* are ascending neurons. The descending (type 3) and ascending cell types (type 4 and type 5) and the median dorsal fibre (MDF) with putative origin in the smaller RGR1 neurons are restricted to the VNC and may thus serve plurisegmental modulatory functions. A modulation of visual inputs cannot be ruled out because of the existence of plexus structures in the ganglionic neuropils of the eyestalk and protocerebrum. In decapods, pronounced neuromodulatory effects of RPCH are known for neuronal circuits in the stomatogastric ganglion (Nusbaum & Marder 1986; Dickinson *et al.* 1989, 1990) and the circuitry controlling swimmeret rhythms (Sherff & Mulloney 1991). However, no information is available about comparable functions of peptidergic interneurons in isopods.

5. CONCLUDING REMARKS

CHH-immunoreactive cells in the CNS of isopods and decapods have been found exclusively in similar neurosecretory pathways supplying the SG. However, the term 'isopod X-organ' seems to be applicable only to these cells but not to the PDH-immunoreactive and the RPCH-immunoreactive cells, since their distributions and pathways in isopods differ considerably from those of comparable decapod analogues. Regarding the PDH-immunoreactive and RPCH-immunoreactive neurons, we have to distinguish between two classes of neurons in both malacostracan suborders. One class comprises classical neurosecretory neurons projecting to neurohaemal organs, the SG or the LCNP, respectively. The second class comprises the majority of immunoreactive neurons, namely centrally branching interneurons in which the neuropeptides may serve as neurotransmitters or neuromodulators.

Our comparative analysis of three different peptidergic systems in the CNS of *Oniscus asellus* provides the structural basis of identified neurons for answering evolutionary questions on the distribution and functional aspects of peptide neurohormones. The high specificity of the anti-CHH and the anti-PDH antisera indicates the presence of neuropeptides in isopods that are closely related to those of the decapods.

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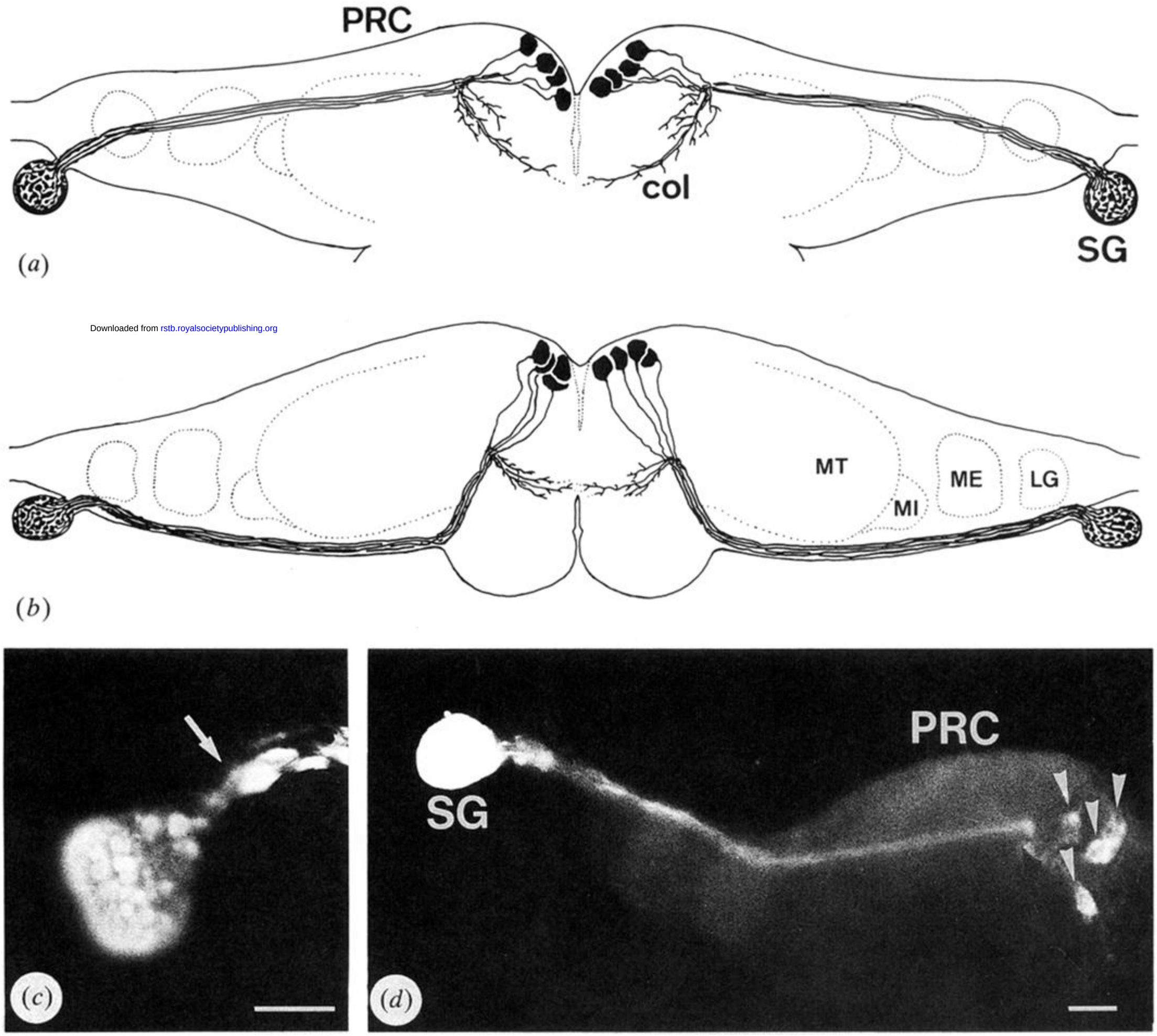
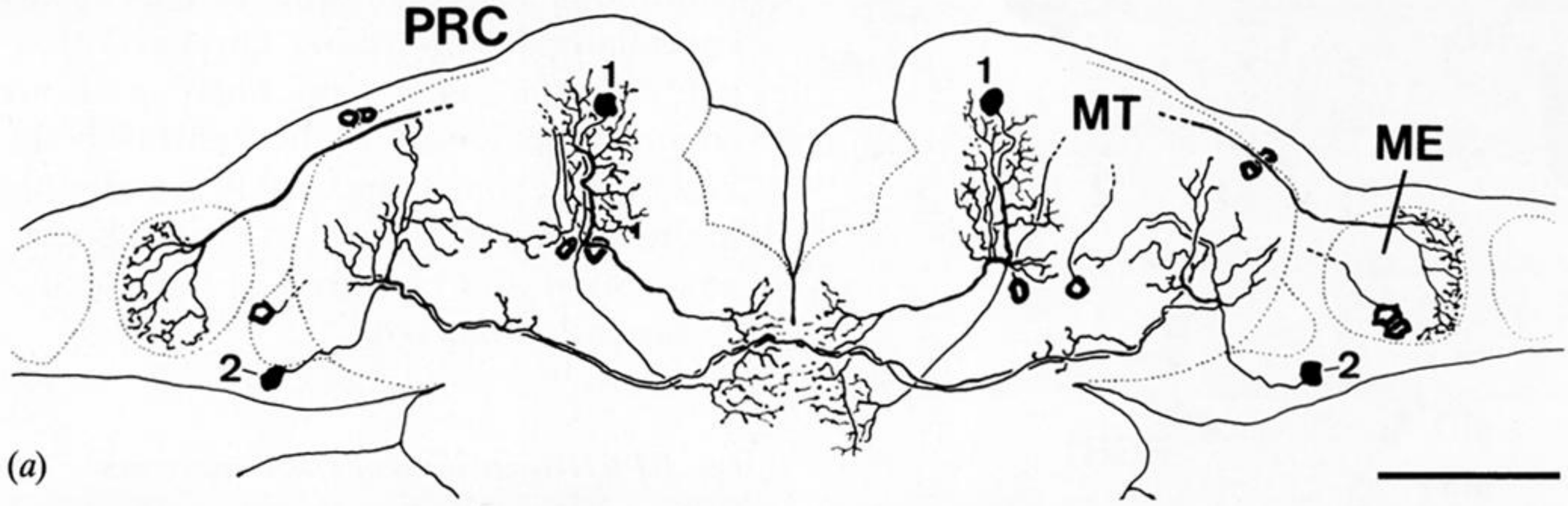
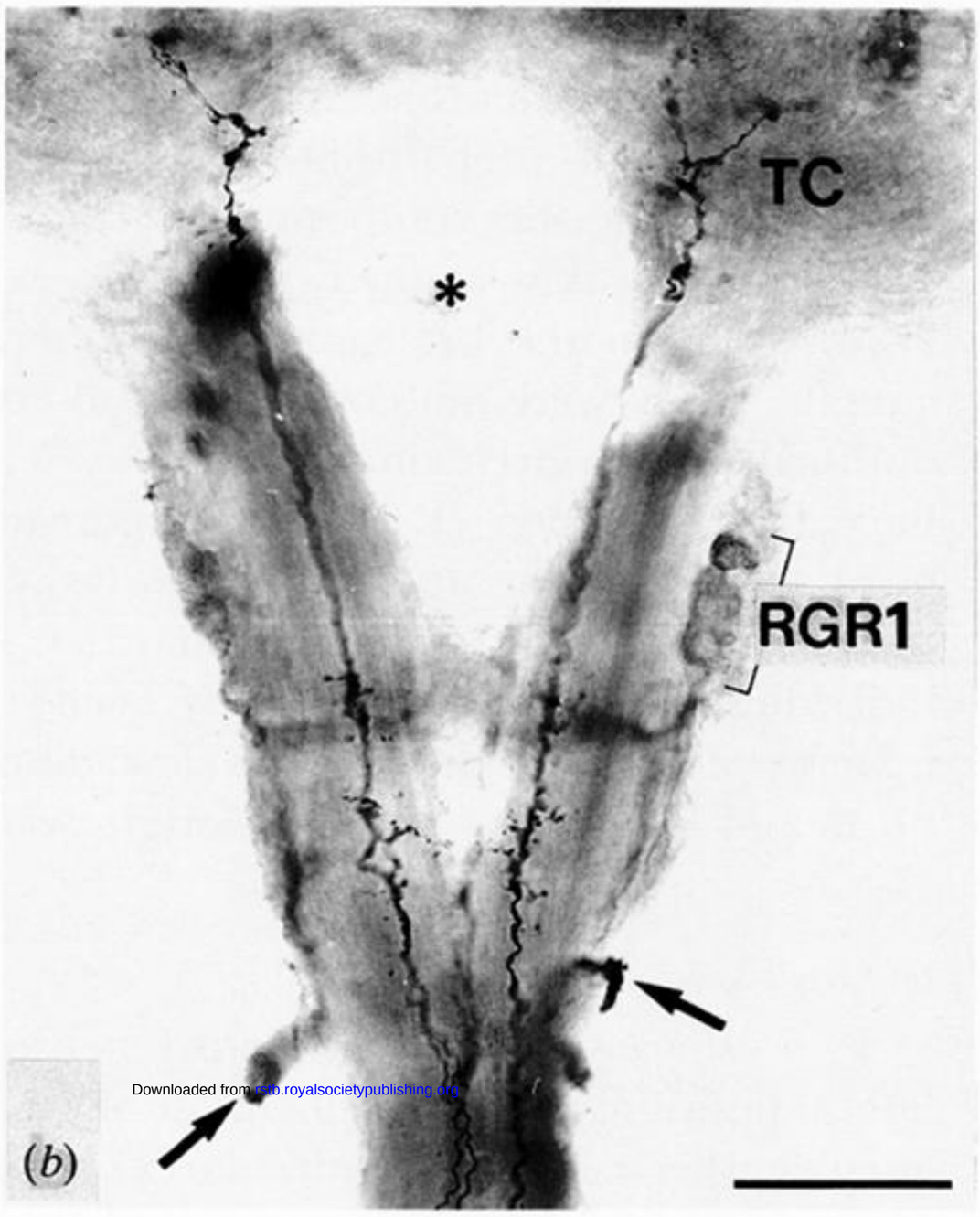
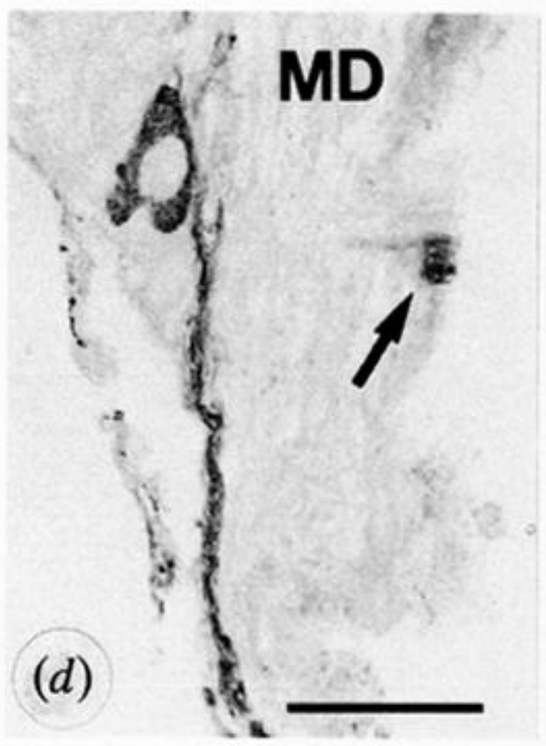


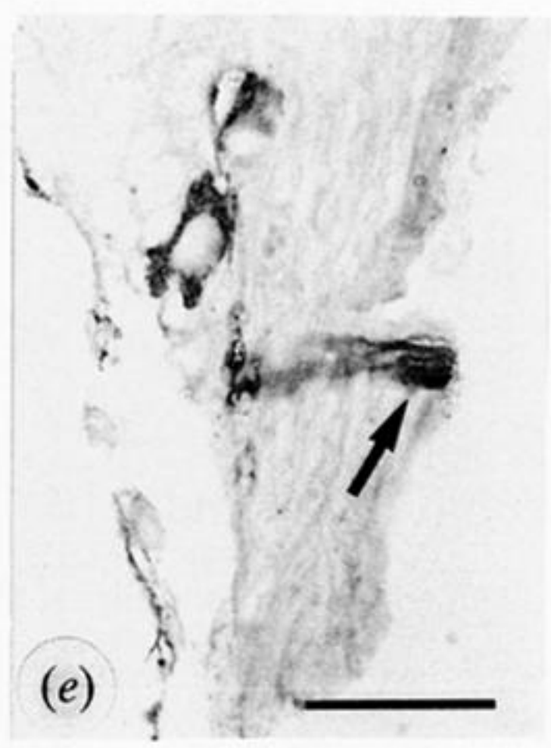
Figure 1. Semidiagrammatic drawing of eight CHH-immunoreactive neurons in a classical neurosecretory pathway of the protocerebrum (PRC) of *Oniscus asellus* as depicted from whole-mount preparations. (*a*) Frontal view. (*b*) Dorsal view. (*c*) Sinus gland (SG) at the posterior lateral margin of the optic lobe; note the bulbous varicosities (arrow) and the abundant terminal axon swellings. (*d*) Perikarya in the isopod medulla terminalis X-organ equivalent and the axon tract to the SG of a hemibrain; note the strong immunoreactivity of the SG in contrast to the faint staining of the tract and the somata. Whole-mount immunofluorescence preparations in (*c*,*d*). Scale bars: 50 µm.

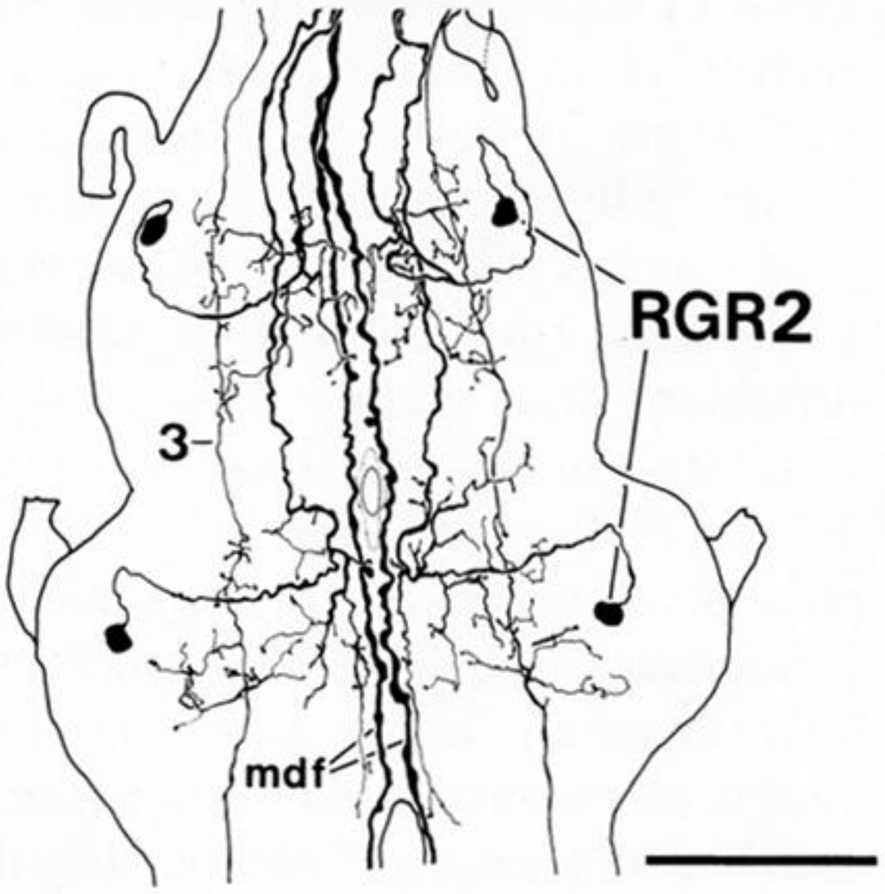
Figure 2. PDH-immunoreactive neurons in the CNS of *Oniscus asellus*. (*a*) Composite diagram of the brain (frontal view) showing three identified groups of neurons (PGR1–3) and their projection patterns as depicted from whole-mount preparations. PGR1 neurons are shown in the right PRC hemisphere, PGR2 and PGR3 neurons in the left. Arrowheads indicate the proposed origin of the fibres shown in (*c*) (cf, commissural fibre; mp, median plexus). (*b*) Camera lucida drawing of all neurons of a left PRC and optic lobe (LO; posterior view; whole-mount preparation). Note the three PGR1 axons entering the SG (arrow), the central arborization of proximal PGR3 axons in the medulla terminalis (asterisk) and the commissural fibre (cf). (*c*) Schematic drawing of two descending fibres in the ventral nerve cord (VNC) which apparently belong to the PGR2 neurons. Note the collateral branchings. No immunopositive perikarya occur in the VNC. (*d*) Sinus gland showing small and sparsely distributed immunopositive axon terminals. Note two PGR3 axons passing through the optic nerve (NO). (*e*) Projections and fibre tracts within a left PRC (frontal view). Note the axon bundle running directly from the mp to the sinus gland (SG) and PGR3 fibres extending into the optic nerve (arrowheads). (*f*) Neuron groups in the median PRC. Note the commissural fibres (arrow), the median plexus (mp), and the lateral plexus of the medulla externa (ME). Whole-mount immunofluorescence preparations in (*d*)–(*f*). Scale bars: (*b*,*d*) 50 µm; (*e*,*f*) 100 µm.











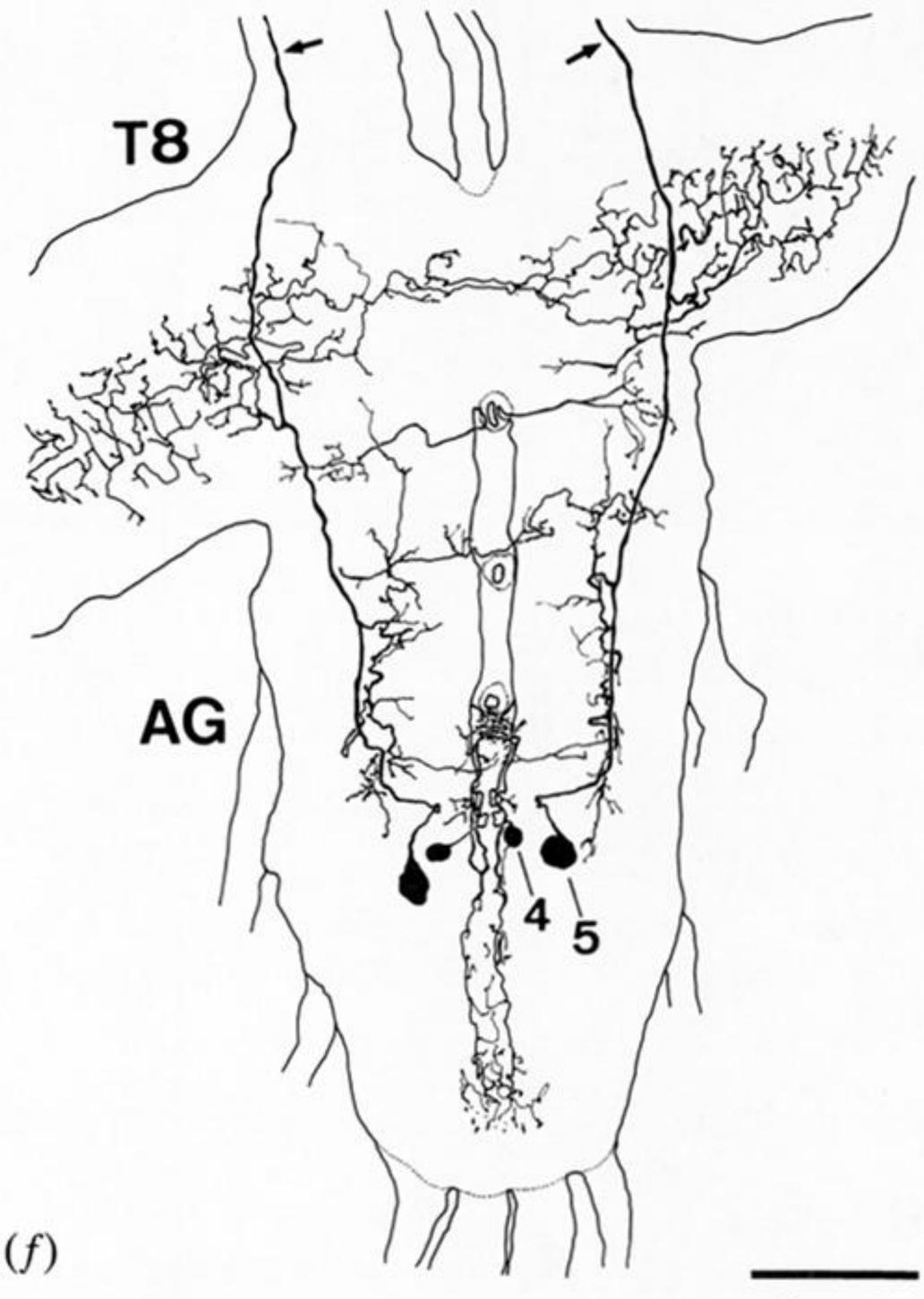
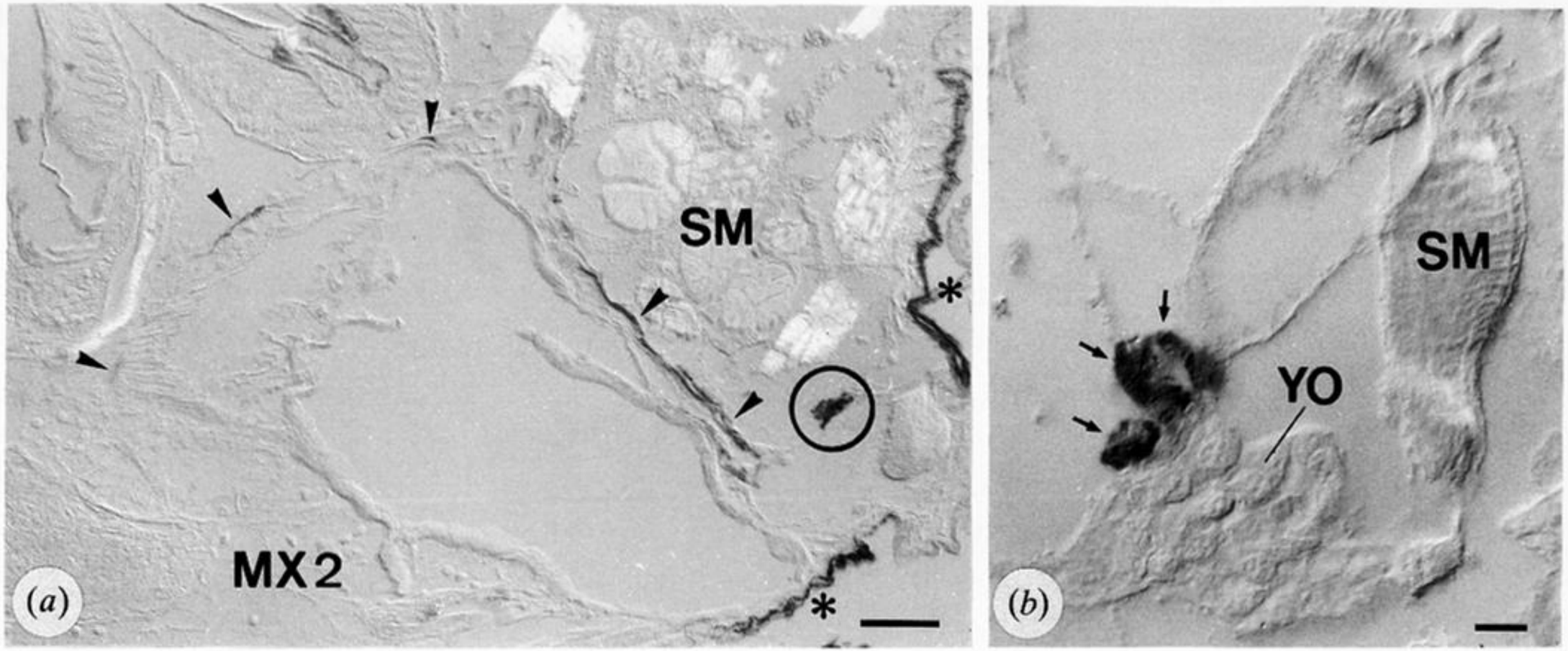


Figure 4. RPCH-immunoreactive neurons in the CNS of *Oniscus asellus*. (*a*) Pathways of type 1 and type 2 neurons and occasionally occurring weakly stained perikarya (unfilled perikarya) in the PRC (frontal view; camera lucida drawing of a whole-mount preparation). (*b*) Weakly stained perikarya of RGR1 neurons projecting into the thick dorsal nerves of MX2 (arrows) leading to the lateral cephalic nerve plexus (not shown), and ascending pathways of RGR2 axons to the tritocerebrum (TC; dorsal view of a PAP-stained whole-mount preparation; compare with (*c*)). Asterisk indicates the orifice of the oesophagus. (*c*) Camera lucida drawing of the RGR2 neurons in the MX2 and MXP following caudally upon (*b*) (same whole mount as in (*b*)). Note the ipsilateral projection patterns. (*d*,*e*) Perikarya of RGR1 neurons and their processes in consecutive PAP-stained longitudinal sections through the mandibular ganglion (MD). Arrows point to the central projections. (*f*) Median type 4 and ascending type 5 neurons (arrows) of the fused abdominal ganglia complex (AG; camera lucida drawing of a PAP-stained whole mount). Note that the small type 4 neurons are restricted to the abdominal ganglia. Scale bars: (*a*,*b*,*c*,*f*) 100 μm; (*d*,*e*) 25 μm.



igure 5. Peripheral RPCH-immunoreactive structures in the right dorsal nerve of the MX2 of *Oniscus asellus* consist f axons ((*a*), arrowheads) of RGR1 neurons supplying the lateral cephalic nerve plexus (LCNP; encircled) in the vicinity f the ventral lateral integument (asterisks) and somatic muscles (SM). Some of these fibres pass the Y-organ (YO), hich is closely attached to the LCNP (*b*), or form endings next to the surface of this nerve plexus (arrows). PAP-stained araplast cross sections through the whole head. Scale bars: (*a*) 50 µm; (*b*) 10 µm.