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Review

Insect ion transport peptides are derived from alternatively spliced genes and differentially expressed in the central and peripheral nervous system

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Summary

Insect ionic and fluid homeostasis relies upon the Malpighian tubules (MT) and different hindgut compartments. Primary urine formed in MTs is finally modified by ion, solute and water reabsorptive processes primarily in the hindgut under the control of several large peptide hormones. One of these, the ion transport peptide (ITP), is a chloride transport-stimulating and acid secretion-inhibiting hormone similar to crustacean hyperglycaemic hormones (CHHs). In locusts, moths and fruit flies, ITP together with the slightly longer ITPL isoforms, inactive in hindgut bioassays, arise by alternative splicing from very similar *itp* genes. ITP and ITPL are differentially distributed in (1) pars lateralis/retrocerebral complex neurosecretory cells (NSCs) containing both splice forms, (2) interneurons with either one of the splice forms, (3) hindgut-innervating abdominal ITP neurons (in *Drosophila* only), and (4) intrinsic, putative sensory NSCs in peripheral neurohaemal perisympathetic/perivisceral organs or transverse nerves (usually containing ITPL). Both splice forms occur as hormones released into the haemolymph in response to feeding or stress stimuli. ITPL mainly released from the peripheral NSCs is discussed as a competitive inhibitor (as established *in vitro*) of ITP action on yet to be identified hindgut ITP receptors. Furthermore, some evidence has been provided for possible ecdysis-related functions of ITP and/or ITPL in moths. The comparative data on the highly similar gene, precursor and primary structures and similar differential distributions in insect and crustacean NSCs suggest that CHH/ITP and ITPL neuropeptide-producing cells and their gene products share common phylogenetic ancestry.

Key words: *Carausius morosus*, *Schistocerca gregaria*, *Locusta migratoria*, *Bombyx mori*, *Manduca sexta*, *Drosophila melanogaster*, *Carcinus maenas*, locust, hindgut, ion transport peptide, antidiuretic hormones, alternative splicing, neurosecretory cells, perisympathetic organs, corpus cardiacum, corpus allatum, homeostasis, reabsorption, water uptake.

Introduction

Renal mechanisms controlling ionic and fluid homeostasis in insects make use of the functional unit of the Malpighian tubules (MTs) and the hindgut. Excretion begins by primary urine formation from osmotically obliged water *via* ion and solute secretion into the MTs and is completed by selective reabsorption of water, ions and metabolites in the hindgut. MT and hindgut functions are tightly but independently controlled by neuroendocrine factors. Several diuretic hormones execute the major control of MT functions (Coast, 1996; Coast, 2001; Coast et al., 2002; Coast, 2007; Dow and Davies, 2003; O'Donnell and Spring, 2000; Schooley et al., 2005). Antidiuretic factors (ADFs) act in some insects on parts of the lower MT segments but in most investigated species they act mainly on the hindgut, in particular the ileum and the rectum (see Coast et al., 1999; Coast et al., 2002; Phillips et al., 1996; Phillips et al., 1998a; Phillips et al., 1998b; Quinlan et al., 1997; Spring et al., 1978; Vietinghoff, 1966). The composition of the final excreta is generally determined by the hindgut except during rapid diuresis in blood-sucking insects, and 'terrestrial insects owe much of their success to their ability to recover virtually all the water' from the flow of primary excretory fluid as already pinpointed by Maddrell (Maddrell, 1981) in early work on the functional design of the insect excretory system. Moreover, the hindgut has important functions in controlling ion (Na^+, Cl^-, K^+) and solute (e.g. proline) uptake, and acid and ammonia secretion, but is separated in ileal and rectal compartments in some insects such as locusts (Audsley et al., 1994; Phillips and Audsley, 1995).

Early bioassay work measuring changes in transepithelial potential or short-circuit current (I_{sc}) upon voltage clamping of the transepithelial potential has shown that ion and fluid reabsorption by hindgut tissues is enhanced by ADFs in extracts of central nervous systems (CNS) and brain–retrocerebral complexes, i.e. mainly pars intercerebralis neurosecretory cells (NSCs) and corpora cardiaca (CC), and in haemolymph of several insects (Spring et al., 1978; Spring and Phillips, 1980a; Spring and Phillips, 1980b; Spring and Phillips, 1980c). Antidiuretic effects vary for some peptides depending upon whether homologous or heterologous bioassay systems using specialised 'fluid-recycling' cryptonephric complexes or simple hindguts have been employed. Peptidic or proteinaceous factors have been detected in the brain and CC and in the ventral nerve cord (VNC; e.g. locust abdominal ganglia 4 to 7) (Audsley and Phillips, 1990) that dose-dependently stimulate ileal I_{sc} and water fluxes (Lechleitner et al., 1989b; Lechleitner and Phillips, 1989). These physiological events are known to be driven by an apical chloride pump electrically coupled predominantly to potassium but also to sodium as passive followers (Phillips et al., 1986; Phillips et al., 1996; Phillips et al., 1998a), the first description of which became a recent JEB citation classic (Bradley, 2008). Among the different brain and CC factors were heat stable and acid labile compounds, but in the VNC only heat and acid labile factors were detected by bioassay. At least three

different classes of large (>8 kDa) peptidic ADFs have been isolated from locusts: the neuroparsins (Fournier et al., 1987; Girardie et al., 1987; Girardie et al., 1989; Girardie et al., 1990), chloride transport-stimulating hormone (CTSH) (Phillips et al., 1980) and ion transport peptide (ITP). Whereas the function of neuroparsins as ADFs has been questioned (Coast et al., 2002), the antidiuretic effects of CTSH and ITP on the locust hindgut are well documented (Coast et al., 1999; Phillips et al., 1996). By use of chromatographic techniques, Audsley and colleagues (Audsley et al., 1992) discriminated three different bioactive compounds in crude CC extracts from the desert locust *Schistocerca gregaria* (Forskål 1775). The first was SchgrITP and the second a more hydrophobic factor. Both were preferentially active on ileal I_{sc} and fluid reabsorption rate (J_v). A third apparently solute transport-stimulating factor had little effect on ileal I_{sc} but stimulated J_v (Audsley, 1991). Whereas the acid labile CTSH acting preferentially on rectal I_{sc} (Phillips et al., 1980) in the desert locust is still to be identified, the only well investigated factor preferentially acting on the ileum I_{sc} is SchgrITP, which is stable in acidic extraction media. This peptide was first isolated and characterised by Audsley and colleagues (Audsley et al., 1992) (see below) and found to be closely related to crustacean hyperglycaemic hormones (CHHs). SchgrITP causes only a submaximal stimulation (40%) of rectal Cl^- transport.

Liao and colleagues (Liao et al., 2000) isolated two ADFs (ADF-A and ADF-B) from brain–CC/corpora allata (CA) complexes of *Manduca sexta* (L.) by use of an everted rectal sac bioassay of larval cryptonephric complexes. Only the slightly more potent ManseADF-B has been investigated in more detail. Its antidiuretic effect was insensitive to specific blockers of proton-pumping vacuolar ATPase, cation/H^+ antiport, and $Na^+/K^+/2Cl^-$ co-transport, all functional elements indispensable for the known paradoxical antidiuretic effects of a diuretic hormone (ManseDH) (Audsley et al., 1993) in cryptonephric complexes. Removal of Cl^- from the lumen side, two different specific Cl^- channel blockers and a specific inhibitor of protein kinase A abolished fluid reabsorption of everted rectal sacs, indicating that the actions of ManseADF-B are clearly Cl^- dependent and are likely to be mediated by cAMP-dependent protein kinase A. However, to again rule out the effects of ManseDH, which probably stimulates $Na^+/K^+/2Cl^-$ co-transport *via* cAMP (Audsley et al., 1993), forskolin (an adenylyl cyclase activator) combined with a co-transport blocker was applied. This mimicked the ManseADF-B action, which was, therefore, assumed to take place in the true rectal epithelium rather than in the cryptonephric compartments of the cryptonephric complexes. The latter are considered targets of ManseDH since they contain the MT elements. This dissection of physiological and pharmacological effects on a cAMP-mediated Cl^- transport similar to that in locusts and the fact that the ADFs are most soluble in 80% ethanol led to the assumption that ManseADF-B is a homologue of either CTSH or ITP of locusts (Liao et al., 2000; Schooley et al., 2005).

For more details on present and past knowledge about ITP physiology the reader is referred to a comprehensive review by Coast and colleagues covering in more depth the historical and some structural and physiological details of locust ITPs/longer ITPs (ITPLs) and other ITPs (Coast et al., 2002). The present review deals with gene and mRNA precursor structures and biochemical characterisations, cellular localisations and distributions of ITP and related peptides in hemi- and holometabolous insects that have hitherto been investigated in more detail. Comparison of genes, precursors and primary structures of ITPs and their distributions in the nervous system have revealed striking similarities to those of CHHs. The latter are major members of a large peptide family comprising CHHs with pleiotropic functions, and moult-, vitellogenesis- and gonad-inhibiting hormones with somewhat more restricted functions (Böcking et al., 2002; Chan et al., 2003; Chen et al., 2005; Keller, 1992; King et al., 1999; Lacombe et al., 1999).

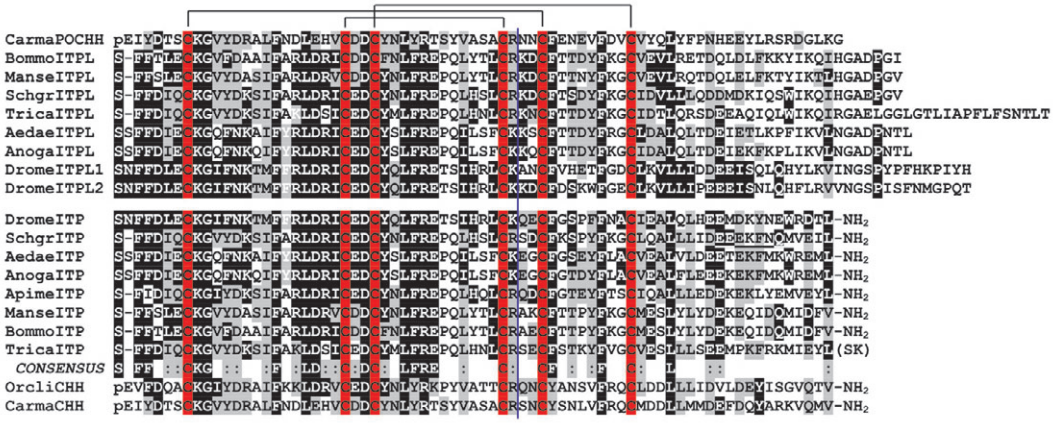

Fig. 1. Alignment of some insect ion transport peptide (ITP) and long ITP (ITPL) sequences in comparison with crustacean hyperglycaemic hormones (CHHs) of shore crab eyestalk ganglia [SGCHHs; *Carcinus maenas*, Carma, P14944 (Kegel et al., 1989); *Orconectes limosus*, Orcli, CAA55308 (Kegel et al., 1991)] and *Carcinus maenas* pericardial organ (PO), CarmaPOCHH (AAG29432) sequences from two crustaceans. The deduced ITP and ITPL sequences are shown for *Manduca sexta* (Manse, AY950500, AY950501), *Bombyx mori* (Bommo, AY950502, AY950503), *Schistocerca gregaria* (Schgr, AAB16822, AAB16823), *Apis mellifera* (Apime, XP001120062), *Aedes aegypti* (Aedae, AY950504, AY950505, AY950506), *Anopheles gambiae* [Anoga, XP313928 (Dai et al., 2007)] and *Tribolium castaneum* (Trica, ABN79657, ABN796578). Sequence similarities and identities are indicated with reference to the ITP/ITPL1-2 of *Drosophila melanogaster* [Drome, ABZ881400, ABZ881401, ABZ881402 (Dircksen et al. 2008)] by grey and black shading, respectively. The consensus estimate includes ITPs/ITPLs only; the colon (:) indicates that only closely related residues are found at this position. Note that at least 14 identical amino acid positions occur mostly in the first parts of the peptide sequences derived from the common exons (as indicated by a vertical blue line behind amino acids 40 or 41) in addition to the six invariable cysteines (red shaded). The latter probably give rise *via* disulphide bridges to three indicated intramolecular loops as so far confirmed only for a few CHHs (e.g. the ones shown here) and a synthetic SchgrITP (King et al., 1999).

Identified peptide and mRNA structures derived from alternatively spliced *chh* and *itp* genes are conserved in crustaceans and insects

In the search for a possible insect hyperglycaemic factor, early attempts were made in the stick insect *Carausius morosus* (Sinety 1901) to identify a putative CHH-like molecule. Strong evidence for a close similarity between this first insect CHH-like peptide (still uncharacterised) and the CHH of the crab *Carcinus maenas* (L.) (CarmaCHH; Fig. 1) was provided by a radioimmunoassay using an antiCarmaCHH serum. The assay showed clear-cut curve parallelism of crude CC extracts and HPLC-purified CarmaCHH (Jaros and Gäde, 1982) long before CarmaCHH had been fully sequenced (Kegel et al., 1989). However, it took 10 more years until the close association of CHHs and insect ITPs became substantiated by the identification of the first partial sequence of 31 amino acids of SchgrITP from *S. gregaria* by Audsley and colleagues (Audsley et al., 1992). Their study gave the first clear-cut hint that SchgrITP is closely related to CHH family peptides (44–59%). However, when tested in the same ileal assay used for the isolation of ITP, CarmaCHH was later found to be inactive, as were head extracts of insects not closely related to locusts from lepidopteran, dipteran, hymenopteran and hemipteran taxa in contrast to those from orthopteran taxa (Meredith et al., 1996). For a CHH this is not surprising considering the fact that they display pronounced species and group specificity when tested in heterologous haemolymph glucose bioassays in different crustaceans (Leuven et al., 1982). In fourth larval instars of the silk moth *Bombyx mori* (L.), a CHH-like peptide similar to SchgrITP was later discovered by cDNA cloning using degenerate primers constructed on the basis of conserved CHH and ITP peptide sequences and called BmCHH-like peptide (BmCHHL= BommoITP; Fig. 1) (Endo et al., 2000).

All CHHs and ITPs/ITPLs have several characteristic features in common. (1) The conformation with six cysteines in the same positions putatively leads to the same three common disulphide bonds. These cysteine bridges are often inferred on the basis of homology but were in fact assigned only in a few studies by tryptic fragmentation of native (or synthetic) non-reduced peptides followed by Edman degradation and/or mass spectrometrical analysis (e.g. Dircksen et al., 2001; Kegel et al., 1989; Kegel et al., 1991; King et al., 1999) (see Fig. 1). (2) The normal length of 72 amino acids for crustacean CHHs is found in many insect ITPs except for dipteran species, which have one more N-terminal amino acid (Ser or Asn in position 2, i.e. 73 amino acids in length). (3) All CHHs and ITPs are C-terminally amidated, which may protect them from carboxypeptidase degradation. (4) N-terminal pyro-Glu, a modification known to protect peptides against aminopeptidase degradation, is a further important structural determinant that is clearly a distinctive difference between most CHHs and all hitherto known ITPs, with the exception of some shrimp CHHs (Chen et al., 2005). (5) The presence of aromatic amino acids (Phe or Tyr) in position 3 (or positions 2, 4 or 3 in dipteran ITPs) of the N-terminal putative α-helix appears to be a very conserved feature that is important for the biological activity of both CHHs and ITPs (Gu et al., 2000; Katayama et al., 2003; Katayama and Nagasawa, 2004; Mosco et al., 2008; Zhao et al., 2005). (6) The highest consensus of amino acid identities or close similarities is restricted to a core structure of the first 40 or 41 amino acids, containing two out of five important characteristic structural motifs in CHHs and ITPs embraced by the probably conserved cystine-bound loops I and II (Chen et al., 2005; Drexler et al., 2007; Lacombe et al., 1999) (Fig. 1).

Meredith and colleagues were the first to discover two structurally closely related mRNAs leading to a short and a long isoform of SchgrITP (SchgrITP and SchgrITPL) by cDNA cloning (Fig. 1, Fig. 2A) (Meredith et al., 1996). It became obvious that the first part of the precursor mRNA of SchgrITPL was identical to the first encoded 40 amino acids of SchgrITP but the rest of the open reading frame (ORF) up to the stop codon was very different, leading to a four amino acid longer peptide with a free carboxy-terminus (134 amino acid long prepropeptide). However, intriguingly, codons encoding the second part of SchgrITP appeared in the 3′-UTR of the SchgrITPL precursor, which up to the 3′-end was otherwise identical to that of the SchgrITP mRNA, and made this different second part of the SchgrITPL peptide look like a stretch called the ‘insert’ by the authors (Fig. 2A). Nearly identical ITP and ITPL mRNAs were found in the migratory locust *Locusta migratoria* (L.), which encoded peptides that were essentially identical to SchgrITP and SchgrITPL with the one exception of D66 *vs* E66 (Macins et al., 1999). These observations gave a first clear-cut hint for the assumption of alternative splicing of locust *itp* gene products later found to occur as a characteristic feature in several *chh* genes (Dircksen et al., 2001; Chen et al., 2004) and *itp* genes (Dai et al., 2007; Dircksen et al., 2008) (Figs 1 and 2). In fact, Dircksen and colleagues (Dircksen et al., 2001) were the first to show that the 72 amino acid-long amidated CarmaCHH from the classical X-organ sinus gland system of the shore crab eyestalk ganglia (CarmaSGCHH) and another slightly longer CHH-like isoform in the intrinsic cells of the neurohaemal pericardial organs (CarmaPOCHH) arise from alternative splicing of pre-mRNAs and differential expression of mRNAs derived from the same *chh* gene (Fig. 1, Fig. 2B). Several crustaceans including the shore crab have been shown to contain multiple *chh* genes that occur in tandem arrangements clustered on the same chromosome and may have arisen from multiple gene duplications during the course of evolution, which further complicates analysis of their messages (Chan et al., 2003; Gu and Chan, 1998). Surprisingly, several variants of mRNAs have been found in the shore crab, most of which definitely did not lead to a translated product and are, thus, of unknown function (Dircksen et al., 2001), a finding recently corroborated by studies on other crabs. These studies stated that preferentially long POCHH-encoding mRNAs occur in several other tissues but no expressed peptides could be found (Chung and Zmora, 2008; Tsai et al., 2008).

In insects, thus far only single copies of *itp* genes have been found. Experimental evidence for the structure of *itp* genes and/or for the true existence of their mRNA products is available only for the lepidopterans *M. sexta* and *B. mori* (Dai et al., 2007; Drexler et al., 2007) and for the dipterans *Aedes aegypti* (L.) and *Drosophila melanogaster* (Meigen 1830) (Dai et al., 2007; Dircksen et al., 2008); other similar provisional *itp* gene-derived precursor and peptide structures, e.g. in the malaria mosquito *Anopheles gambiae* (Giles 1902) (Anoga) and the red flour beetle *Tribolium castaneum* (Herbst 1797), have been revealed by bioinformatic data mining. Whilst the currently annotated versions of the Anoga *itp* gene may comprise three exons only (ENSANGT00000021718) (Dircksen et al., 2008) similar to the Manse *itp* gene (Fig. 2B), the *T. castaneum* genome contains a TricaITPL and a putative TricaITP-like sequence (Li et al., 2008), though not fully conforming to the currently annotated four-exon Trica *itp* gene model (gene 657659). The former peptide is currently the longest known ITPL isoform, but the putative TricaITP sequence may still be incorrect as the C-terminus is two amino acids longer than in all other ITPs and does not contain an expected typical amidation site. The latter example shows how important it is to perform ‘postgenomic verification’ of

mRNAs (especially of their 5′- and 3′-ends) by cDNA cloning and of the deduced peptides by biochemical analysis. In fact, the definite occurrence and biochemical identity of ITP and ITPL peptides has thus far only been proven in the cases of the desert locust and the fruit fly ITPs (Audsley et al., 1992; Audsley et al., 2006; Dircksen et al., 2008).

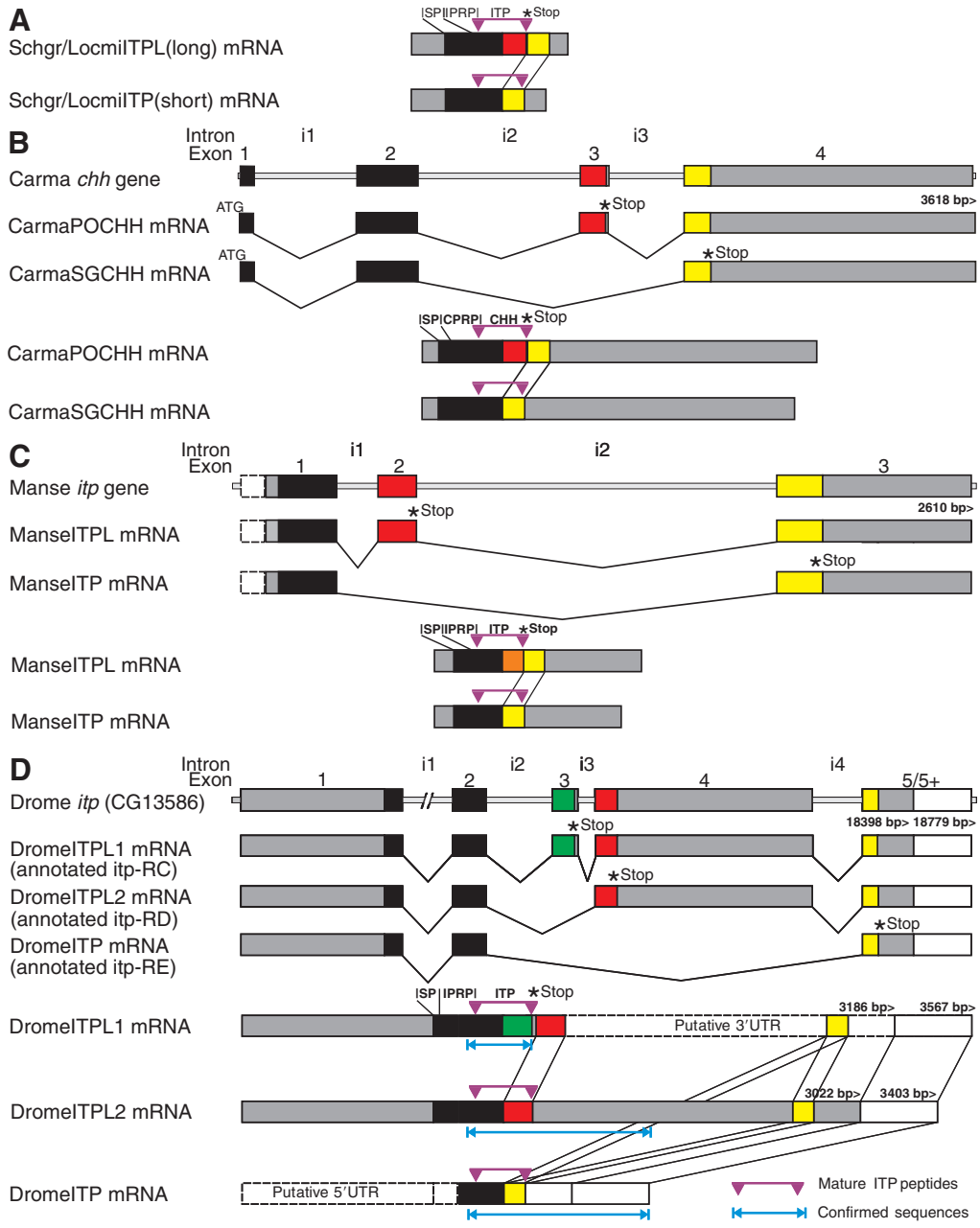

Fig. 2. Schematic representation of highly conserved CHH/ITP gene structures containing exons arranged in tandem leading to derived alternative mRNA splice forms that encode the common (black) and the distinctive parts of long (red, green; POCHH/ITPL) and short (yellow; SGCHH/ITP) peptide isoforms (not exactly to scale). Note the occurrence of exons common to the first parts (black) in 5′-positions of the open reading frames (ORFs) within the derived mRNAs, and of the short splice form-specific ORF parts (yellow) always in the 3′-untranslated regions (UTRs) of the long splice forms. Also indicated are 5′- and 3′-UTRs (grey) and other proposed non-coding (white) regions of exons. Mature ITP peptides are indicated by magenta triangle lines. (A) Structures of the two mRNAs of short and long locust ITP/ITPL putative splice forms. Almost identical mRNAs lead to identical SchgrITP/LocmiITP in the two locust species, but SchgrIPTL and LocmiITPL differ by one amino acid (D66 to E66) (Macins et al., 1999; Meredith et al., 1996). (B) The *chh* gene of *C. maenas* has four exons leading to two identified mRNAs differentially expressed in the X-organ sinus gland system (CarmaSGCHH) or pericardial organ cells (CarmaPOCHH) (Dircksen et al., 2001). (C) The three exon Manse *itp* gene model and the derived and mature ManseITP/ITPL mRNA structures (Dai et al., 2007). (D) The current and revised five exon Drome *itp* gene model (CG13586, cf. current annotation) and the proposed and partially confirmed (blue arrowed lines) derived two long DromeITPL1,2 and one short DromeITP mRNA splice forms with putative 5′- and 3′-UTRs; intron 1 is drawn interrupted because it is much larger (14 kb) than shown here. Note that corresponding splice form-specific ORF parts of DromeITPL2 (red) and DromeITP mRNAs (yellow) are contained in the 3′-UTRs of the DromeITPL1 and DromeITPL2 mRNAs, respectively [after Dircksen et al., with permission (Dircksen et al., 2008)]. SP, signal peptide; CPRP/IPRP, CHH/ITP precursor-related peptide.

The principal characteristics of *chh* and *itp* genes appear to be highly conserved in crustaceans and insects. At present, a four exon *chh/itp* gene model, as discussed previously by Chen and colleagues (Chen et al., 2005), seems to emerge as the most likely unifying concept. Nevertheless, the probably 5′-incomplete three-exon gene models for the Manse *itp* and Anoga *itp* genes and recently detected ambiguities with regard to the true existence of the first exon in the current five exon Drome *itp* gene model [as discussed before (Dircksen et al., 2008)] among others may need to be clarified first. Definitely, all hitherto investigated and predicted cases of *chh/itp* genes contain one or two common 5′-exons leading to mRNAs identically coding for the first 40–41 amino acid peptide parts of both the long and short CHH/ITP isoforms. These exons are in most cases followed by two exons (three in the case of *D. melanogaster*) leading to the mRNA encoding the 'second parts' (amino acids 41+ or 42+) of the long and the short CHH/ITP isoforms (Fig. 2A–D). The short isoforms are thus far always encoded by the very last exon, and their splice sites are apparently not easily recognised by automated annotation software. Furthermore, a still unresolved and enigmatic issue is the occurrence and functional significance of normally co-processed so-called precursor-related peptides for CHHs and ITPs (CPRPs and IPRPs) that in most cases are found in tissue extracts or co-released (e.g. Dircksen et al., 2001; Huybrechts et al., 2003; Tensen et al., 1991; Toullec et al., 2006; Wilcockson et al., 2002) (Fig. 2). Their messages within the 'common exons' always precede those of the CHH or ITP messages, are separated from the latter by typical dibasic cleavage sites, but may differ tremendously in peptide size (7–54 amino acids) among the investigated species from the different arthropod taxa.

Localisation of CHH-like and ITP-like gene products and peptides in insects throughout postembryonic development

The distribution of distinct CHH/ITP- and ITPL-immunoreactive neurons and/or neuronal cell bodies detected by *in situ* hybridisation with *itp* gene-specific probes (which thus far cannot distinguish between ITP/ITPL splice forms) is restricted to four principal neuron types in the investigated insects. These are in all species (1) a few NSCs in the pars lateralis of the protocerebrum with projections to the retrocerebral complex (CC/CA; with *C. morosus* as a possible exception, see below), but in some insects also (2) interneurons in the brain and ventral nerve cord (suboesophageal, thoracic and abdominal ganglia; SOG, TG, AG), (3) efferent neurons in terminal AGs innervating the hindgut and (4) neurosecretory cells associated with neurohaemal organs of the peripheral nervous system (PNS). This distribution of NSCs is similar to the occurrence of CHH neurons in the X-organ sinus gland system of crustacean eyestalk ganglia and neurohaemal pericardial organs. However, ITP/ITPL-immunoreactive or *in situ* hybridising cell structures have thus far not been found anywhere but the insect CNS and PNS, especially not in gut tissues. This is contrary to the situation in shore crabs where CarmaSGCHH transiently occurs in thousands of endocrine cells of the ectodermal foreguts and hindguts during the preparations for moulting. CarmaSGCHH is released in enormous amounts from these cells during the time of ecdysis, immediately after or during which the cells shut down their production (Chung et al., 1999) (see also below, and cf. Table 1).

Even before the primary structures of any CHH or ITP were known, immunocytochemistry using a crab antiCarmaCHH serum helped, for the first time, to localise CHH-like peptide immunoreactivity in about 50 NSCs in the pars intercerebralis of the stick insect *C. morosus* (Jaros and Gäde, 1982). The authors stated that the cells probably projected to terminals in the CC but not in the CA and substantiated this finding by radioimmunoassay analysis of brain and CC extracts. No CHH immunoreactivity was detected in other parts of the CNS and VNC. Our first attempts to localise CHH/ITP-like peptides in *L. migratoria* by *in situ* hybridisation resulted in the detection of OrcliCHH immunoreactivity in about 20 lateral NSCs with projections to the retrocerebral complex (CC and CA) and in about 10–12 intrinsic peripheral NSCs in the link and transverse nerve-associated dorsal perisympathetic organs [LN, TN and dPSOs; terminology according to Dircksen et al. (Dircksen et al., 1991)] of each abdominal segment using an antiserum to purified SGCHH of the spiny cheek crayfish *Orconectes limosus* (Rafinesque 1817) (Keller, 1988) (OrcliSGCHH; Figs 1 and 3; Table 1). Occasionally, single pairs of anterior dorsal lateral OrcliCHH-immunoreactive neurons were detected in abdominal ganglia AG4–AG7 (Dircksen and Heyn, 1998) (Fig. 3C), which may agree with the above-mentioned earlier findings of I_{sc}-stimulating factors in these ganglia (Audsley and Phillips, 1990). Final identification of the *Locusta* CHH/ITP-immunoreactive substances is pending, but western blots of CC/CA extracts using the same antisera as for *in situ* hybridisation indicated molecular sizes close to those of the known LocmiITPs (H.D., unpublished). This study established the CC/CA as a putative release site(s) for circulating ITP neuropeptides in locusts, as dealt with later in more detail (see below). A comprehensive study employed western blots with antisera raised against synthetic full-length SchgrITP and N- and C-terminal peptide haptens (Ring et al., 1998) and an I_{sc} bioassay to investigate the tissue distributions of extractable ITPs and ITPLs in seven orthopteroid insect species (including *L. migratoria*) (Macins et al., 1999), but did not determine the exact cellular localisation of these substances. Brain and/or CC complexes of all orthopteroid insect species contained SchgrITP-immunoreactive, but not SchgrITPL-immunoreactive, substances; extracts from five times as many CC equivalents from blowflies *Neobellieria bullata* (Parker 1916) and lepidopterans *Spodoptera litura* (Fabricius 1775) did not immunoreact at all and also did not show any bioactivity. For the locusts, a previously shown wider tissue distribution of desert locust ITP and ITPL transcripts and peptides (Meredith et al., 1996; Ring et al., 1998) could only partly be supported. For instance, concentrated brain, ileal and rectal tissue samples of *S. gregaria* did not display any SchgrITPL immunoreactivity, in contrast to previous results using reverse transcription polymerase chain reaction (RT-PCR) that had shown the presence of SchgrITPL mRNAs (Meredith et al., 1996). However, a recent immunocytochemical study relying on the close structural similarities of ManseITPL and SchgrITPL peptides (Fig. 1), and antibodies specific to the C-terminal tetradecapeptide of ManseITPL, succeeded in localising SchamITPL-like immunoreactivity in the American bird grasshopper *Schistocerca americana* (Drury 1773), a species closely related to *S. gregaria* (Dai et al., 2007). The authors found about nine dorsolateral ManseITPL-immunoreactive NSCs in the brain and 9–12 pairs of peripheral link nerve- and dPSO-associated neurons in this species, but could not demonstrate any ManseITP-immunoreactive elements. These findings are well in accordance with the RT-PCR results obtained for SchgrITP and SchgrITPL in the brain of *S. gregaria* (Meredith et al., 1996) and our immunocytochemistry results on the distribution of CHH/ITP-like immunoreactivity in the brain and peripheral NSC in *L. migratoria* (Dircksen and Heyn, 1998) (Fig. 3). The latter is supported by the fact that none of our

Table 1. Comparison of neuronal structures detected by peptide family-specific or isoform-specific (in bold) antisera to ITPs, SGCHHs or ITPLs, and POCHHs and by *in situ* hybridisation in the CNS and PNS of insects and a crab

Species, Abs/RNAs	Brain PI/PL	Brain IN	Terminals CC	Terminals CA	SOG	TG1–TG3	AG(TAG)	PNS NSC, LN/TN-PSO/PVO	Hindgut, innervation by efferent neurons
Carausius morosus[1]									
AntiCarmaSGCHH ICC	*ca.* 50 PI	–	+	–	–	–	–	n.d.	n.d.
Locusta migratoria[2]									
AntiOrcliSGCHH ICC	*ca.* 20 PL	–	+	+	–	–	1 dl IN AG4–AG7	10–12 per TG/AG seg., +	–
Schistocerca americana[3]									
AntiMasITPL ICC	9 PL	–	n.d.	n.d.	n.d.	n.d.	n.d.	9–12 (per AG seg.?), +	n.d.
Bombyx mori[3,4]									
MasITP–ISH, L4[4]	5–6 PL(Ia_2)	–			n.d.	n.d.	n.d.	n.d.	n.d.
AntiMasITP, larvae[3]	6 PL(Ia_2)	–	+	n.d.	–	–	–	–	n.d.
AntiMasITPL, larvae[3]	6 PL(Ia_2)	–	–	n.d.	–	1/all TG	1/all AG	L1/all TG2–AG9 seg., +	n.d.
Manduca sexta[3,5]									
AntiCarmaCHH L5, P, Ad[5]	5 PL(Ia_2)	–	+	(+)	5, 3, 0, 0 IN L, eP, lP, Ad	2, 0, 2–4, 0 L, eP, lP, Ad	3–4 all, 3 AG3, 0 L, eP, lP, Ad	0, + IN L5, P, Ad	n.d.
ITP/ITPL–ISH, L2, 4, P, Ad[3]	5 PL(Ia_2)	3–5L, Ad ls 2Ad dl			–	1a, 1–2p all TG	–	1 per seg. in L2–AG 2–7	n.d.
AntiMasITP, L2, 4, P, Ad[3]	5 PL(Ia_2)	3–5L, Ad ls –	+	+	–	–	–	–	–
AntiMasITPL, L2, 4, P, Ad[3]	5 PL(Ia_2)	– 2Ad dl	–	–	–	1a, 1–2p dl all TG	1a dl, all AG, 1 VMN AG2–6	1 per seg. in L2–AG 2–7	–
Drosophila melanogaster[6]									
AntiOrcliSGCHH ICC	4 PL ipc-1	2a	+	+	–	–	1 VMN AG1–AG3	n.d.	–
DrmITP/ITPL2–ISH, L3	4 PL ipc-1	–	–	–	1L3 isog	–	1 iag	n.d.	n.d.
DrmITP/ITPL2–ISH, Ad	4 PL ipc-1	4ipc-2, -3	–	–	–	n.d.	n.d.	n.d.	n.d.
AntiScgITP–C1, L1–3, P	4 PL ipc-1	–	+	+	1L1–L3, eP	Ad TG1–3	1–3 iag L1–L3,P	1 ipn LBD per seg. in AG8	From 3 iag L3
AntiScgITP–C1, Ad	4 PL ipc-1	4ipc-2, -3 2ipc-4	+	+	isog	Root NSC	3 iag Ad	1 ipn BpN_6 per seg. AG7	From 3 iag Ad
Species, Abs/RNAs	Eyestalk	Brain IN	SG		SOG	TG1–TG3	AG(TAG)	Pericardial organs	Hindgut, innervation by efferent neurons
Carcinus maenas[7]									
AntiCarmaSGCHH ICC	62–65 XO	–	+		–	–	–	(4ab, 10–15pb NSC)	Endocrine cells only[8]
AntiCarmaPOCHH ICC	–	–	–		–	–	–	4ab, 10–15pb NSC	–

Abbreviations: Abs, antibodies; BpN, peripheral bipolar neuron (Dulcis and Levine 2003); CC/CA, corpora cardiaca/corpora allata; CNS/PNS, central/peripheral nervous system; a/p/d/l/s, anterior/posterior/dorsal/lateral/small interneuron; ICC, immunocytochemistry; ISH, *in situ* hybridisation; IN, interneuron; ITP/ITPL, ion transport peptide short and long isoforms; ipc/isog/iag/ipn, Drm/TP-immunoreactive protocerebral/SOG/AG/peripheral neurons; n.d., not determined; LN/TN-PSO/PVO, link/transverse nerve perisympathetic organs/perivisceral organs; L1, peripheral NS-L1 neuron (Davis et al., 1993); Lx/P/Ad, larval stage X/(eP early, lP late) pupa/adult; LBD, peripheral 'lateral bipolar dendrite' neuron (Bodmer and Jan, 1987); NSCs, neurosecretory cells; seg., segment; SGCHH and POCHH, sinus gland and pericardial organ crustacean hyperglycaemic hormone isoforms; PI/PL, pars intercerebralis/pars lateralis NSC; SOG/TG/AG(TAG), suboesophageal/thoracic/(terminal) abdominal ganglia; VMN, ventromedial interneuron; XO, X-organ cell bodies; +/–, observed/not observed.

References: [1]Jaros and Gäde (1982); [2]Dircksen and Heyn (1998) and this study; [3]Dai et al. (2007); [4]Endo et al. (2000); [5]Drexler et al. (2007); [6]Dircksen et al. (2008); [7]Dircksen et al. (1988, 2001); [8]Chung et al. (1999).

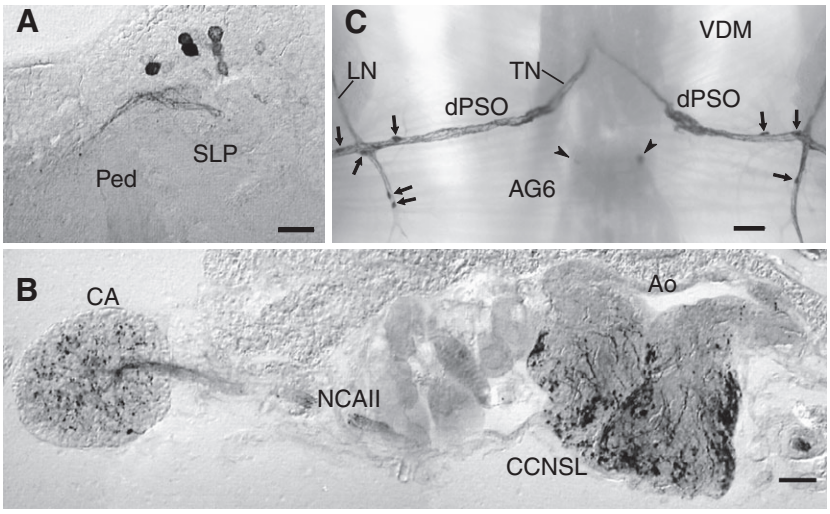

Fig. 3. CHH/ITP-like immunoreactive neuronal structures in the brain–retrocerebral complex and associated with the abdominal peripheral nervous system of *Locusta migratoria*. (A) Pars lateralis NSCs of varied staining intensity in the anterior right brain hemisphere and dendrite-like fibres in the superior lateral protocerebrum (SLP). Ped, pedunculus of the mushroom body. (B) Terminals in the neurosecretory lobe of the corpora cardiaca (CCNSL) and in the corpora allata (CA) arising from the NSCs shown in A. Note the fibres in the nervus corporis cardiaci II (NCAII) which connects the CC and CA. Ao, aorta. (C) Peripheral NSCs (arrows) intrinsic to neurohaemal areas of the distal perisympathetic organs (dPSOs) located at the crossing of the transverse and link nerves (TN, LN) usually attached to the ventral diaphragm muscles (VDMs); the structures are overlying the sixth abdominal ganglion (AG6) that also contains one pair of immunostained dorsal lateral putative interneurons (arrowheads). Horizontal 50 μm-thick vibratome sections (in A and B) and whole mount (in C) stained with antiOrcliCHH 1:2000 using the peroxidase–antiperoxidase (PAP) technique. Scale bars: 50 μm, A and B; 100 μm, C.

antisera to crab or crayfish SGCHHs can discriminate between cross-reactive ITP and ITPL isoforms (H.D., unpublished) and could, therefore, perhaps be called 'CHH/ITP-peptide family-specific' antisera (cf. Table 1).

In situ hybridisation was first employed in *B. mori* to localise Bommo *itp* gene transcripts to five to six cells with anterior lateral positions in the protocerebrum of L4 larvae (Endo et al., 2000), but their associations with neurohaemal release sites remained unclear. The latter was demonstrated only recently for the first time in lepidopterans, in fifth instar larvae and adult *M. sexta*. Two independent studies found CarmaCHH-immunoreactive and ManseITP-immunoreactive NSCs in lateral parts of larval and adult moth brain that project to the CC and the CA; the first study also examined the entire CNS of *B. mori* larvae (Dai et al., 2007; Drexler et al., 2007). The latter study also used antisera against SGCHH of *Cancer pagurus* (L.), which gave the same results as antiCarmaCHH. These NSCs are most likely identical to the type-Ia_2 NSCs previously well defined by cobalt backfilling studies (Copenhaver and Truman, 1986; Nijhout, 1975). Co-localisation experiments showed that these NSCs are different from known corazonin-immunoreactive neurons in the same region (Dai et al., 2007). Drexler and colleagues (Drexler et al., 2007) discovered complex developmental changes in the distribution of distinct CarmaCHH-immunoreactive VNC neurons that, however, 'only stain occasionally' during late larval, and early and late pupal stages into the pharate adult stages in SOG, TGs and AGs (Table 1). On the other hand, the numbers and projections of lateral brain type-Ia_2 NSCs to the CC/CA did not change much with the possible exception of faintly labelled NSC and CC/CA projections in late pupae. However, Dai and colleagues (Dai et al., 2007) found ManseITP/ITPL transcripts but only strongly ManseITPL-immunoreactive putative (ascending) interneurons in all TGs and AGs of larvae, pupae and adults of *M. sexta*. No such neurons were seen in the SOG of any stage. In the same study, the authors found that there is actually a very similar distribution of MasITPL-immunoreactive putative interneurons in larvae of *B. mori* (Fig. 4; Table 1). Thus, the possibility cannot be excluded that the antiCarmaCHH and antiManseITPL antibodies may have detected different compounds in the VNC of the *M. sexta* postembryonic stages. Furthermore, the origin of the strongly CarmaCHH-immunoreactive peripheral fibre networks detected in the transverse nerve-associated neurohaemal perivisceral organs (TN-PVOs, homologues of locust dPSOs) was assumed to lie in some median cells of the abdominal ganglia (AG2–AG9; claimed M2/M3 cell types) (Drexler et al., 2007). However, Dai and colleagues (Dai et al., 2007) showed that well known peripheral NSCs, occurring near spiracles (type NS-L1) (Davis et al., 1993), were the source of similar and strongly ManseITPL-immunoreactive TN-PVO networks and terminals. The NS-L1 are known to contain crustacean cardioactive peptide (CCAP) (Davis et al., 1993), and Dai and colleagues (Dai et al., 2007) actually confirmed their identity by showing co-localisation of CCAP and ManseITPL immunoreactivity in these NSCs.

A less complex (at first glance) distribution of DromeITP-immunoreactive neurons was discovered recently in the CNS and PNS of the fruit fly and investigated throughout postembryogenesis (Dircksen et al., 2008). This study used a hapten antiserum against the C-terminal so-called C1-fragment of SchgrITP (DEEEKFNQ) (Ring et al., 1998), which was shown to cross-react with DromeITP. Although similar sequence parts occur in DromeITPL1 and DromeITPL2 (DEEISQLQ and EEEISNLQ, respectively; Fig. 1), this antiserum apparently did not cross-react with these long isoforms, as has now been further ascertained by distribution analyses of the long isoforms using novel isoform-specific antisera (H.D., unpublished; see also below). The authors unsuccessfully analysed embryos, but found in all larval, pupal and adult stages four strongly ITP-immunoreactive neuronal cell types, except for an SOG interneuron type (see below; Table 1). The results were substantiated by the identification of three different mRNAs derived from the Drome *itp* gene CG13586 (see Fig. 2); one of them, which encoded the short DromeITP isoform, was novel. The other two mRNAs (encoding DromeITPL1 and 2) were only provisionally software annotated before and have been changed again in the meantime (see http://flybase.org/reports/FBgn0035023.html). Furthermore, both DromeITP and DromeITPL2 were identified in CNS extracts and by mass spectrometry of ring glands and adult CC/CA, but the DromeITPL1 peptide was still not found. These results were further substantiated by *in situ* hybridisation showing the presence of the mRNAs in ITP-immunoreactive neurons in the CNS of third instar larvae and adult fruit flies (Table 1). In all postembryonic stages, a strongly ITP-immunoreactive and *in situ* hybridisation-positive group of four NSCs (occasionally five) always occurs in the lateral protocerebrum (ipc-1, for ITP-immunoreactive protocerebral) that project to the CC and partly to the CA. These are probably

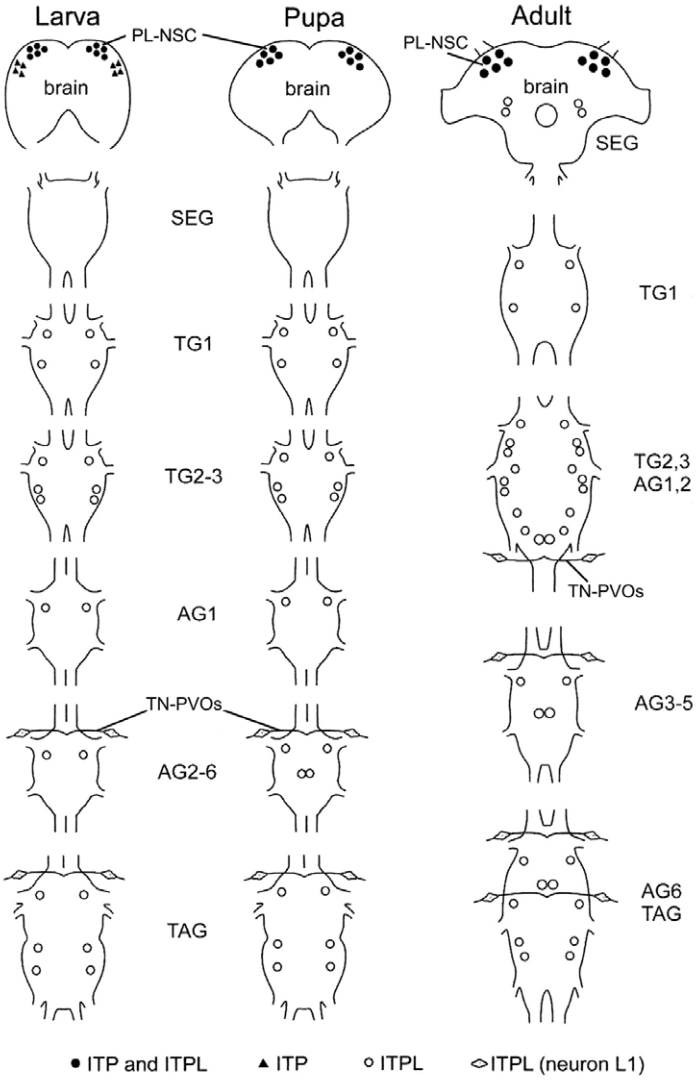

Fig. 4. Distribution scheme of ManseITP- and ManseITPL-expressing central and peripheral neurons of larval, pupal and adult stages of *M. sexta*; the larval part also applies identically to larvae of *B. mori*. ManseITP and ManseITPL occur together in the same pars lateralis (PL-)NSCs. Only ManseITP is in further lateral larval brain interneurons, and again only weakly expressed ManseITPL occurs in other adult brain interneurons. Anterior dorsal lateral interneurons in all VNC ganglia of all developmental stages, some median interneurons in pupal and adult AG2–AG9, and peripheral NS-L1 NSCs in TN-PVOs express ManseITPL, whereas ManseITP is restricted to brain neurons. SEG, suboesophageal ganglion; TG, thoracic ganglia; TAG, terminal abdominal ganglion. Slightly modified from Dai et al., with permission (Dai et al., 2007).

homologues of the previously described pars lateralis NSCs in locusts and moths. Their positions in the brain only change during metamorphosis, when they finally come to lie in more posterior dorsal–lateral positions in the adult brain, from where they innervate the retrocerebral complex (CC and CA) (Fig. 5A,B). In the adult brain, the ipc-1 neurons appear to share an axonal tract system with another neuron type (ipc-2), which may innervate the retrocerebral complex as well and become ITP-immunoreactive only in late pupal stages. These are distinct from another two putative interneuron types (ipc-3 and -4) with restricted projections within the adult brain only (Fig. 5B). From the first larval stage up to the first pupal stages only, a strongly ITP-immunoreactive interneuron (isog) occurs in the SOG with extensive projections in the posterior brain and dorsally down through all VNC neuromeres (Fig. 5). In the terminal abdominal neuromeres its branches overlap with those of one to three efferent ITP-immunoreactive neurons (iag), which innervate the hindgut only. These neurons occur with invariant projection sites from the first larval into the adult stage. Thus, with the exception of the isog neuron, which lose their ITP immunoreactivity in midpupal stages, the larval efferent ipc-1 and iag neuron types are remarkably invariant in number and destination throughout development and metamorphosis. The same appears to be true for another peripheral ITP-immunoreactive putative NSC, the lateral bipolar dendrite (LBD) neuron, found to be ITP-immunoreactive only in the last abdominal segment 8. The LBD neuron is a well known putative sensory cell type in all abdominal segments and is associated with the transverse nerves (Bodmer and Jan, 1987; Gorczyca et al., 1994). It is similar in position to the BpN neurons in adult flies, which in the last abdominal segment are also ITP-immunoreactive. In the transverse nerves, LBD neurons are known to receive contacts from central motoneurons and to reach dorsally up to the alary muscles of the heart (Landgraf and Thor, 2006). The LBD neurons are similar, if not homologous, to the locust dPSO- and the moth PVO-associated (NS-L1) peripheral NSCs. Our most recent preliminary results using novel specific antisera against DromeITP and the two long DromeITPL1,2 isoforms confirmed the distribution of the antiSchgrITP-C1-immunoreactive neurons (as reviewed here), but found DromeITPL1 and DromeITPL2 immunoreactivity only in LBD neurons of at least four different abdominal segments anterior to abdominal segment 8 (H.D., unpublished).

Possible functions of ITPs and ITPLs, and functional morphology of ITP/ITPL neurons

Much has been learned from *in vitro* studies about the effects of ITP and ITPL on the hindgut. Can the derived concepts be confirmed by *in vivo* studies, and are these the only physiological actions of both peptides? As a first step towards answering these questions, Audsley and colleagues (Audsley et al., 2006) undertook an important study to measure and identify the proposed circulating peptides SchgrITP and SchgrITPL in the CC and the haemolymph of fed (0.5–8 min and directly bled thereafter) and starved *S. gregaria*. The authors used enzyme-linked immunosorbant assays (ELISAs) with hapten antisera specifically discriminating SchgrITP (with the above-mentioned C1-antiserum) and SchgrITPL [with antisera raised against the SchgrITPL65–79 fragment; both antisera produced by Ring and colleagues (Ring et al., 1998)]. They found both SchgrITP and SchgrITPL peptides in similar amounts of 117 and 88 fmol per CC, respectively. Assuming 2–5% of material to be released and a total haemolymph volume *ca*. 800 µl for locusts (Couillaud et al., 1987), this could just account for the 4-fold increase in concentration of SchgrITPL detected in the haemolymph of fed *vs* starved locusts (41.5 *vs* 9.6 nmol l^{-1}). Unfortunately, SchgrITP turned out to be much more difficult to measure and reliably identify than SchgrITPL, because it may be degraded much faster than the latter in haemolymph, as suggested by the occurrence of several ITP-immunoreactive chromatographic fractions not co-eluting with an ITP standard. When compared with ITPL, this seems surprising, because ITP is C-terminally amidated, i.e. blocked against carboxypeptidase digestion. In fact, negative results of degradation tests with synthetic SchgrITP and haemolymph *in vitro* seemed to argue against the ITP degradation hypothesis. Nevertheless, haemolymph from fed *vs* starved locusts showed an approximately 80-fold increase (14.2 *vs* 0.17 nmol l^{-1})

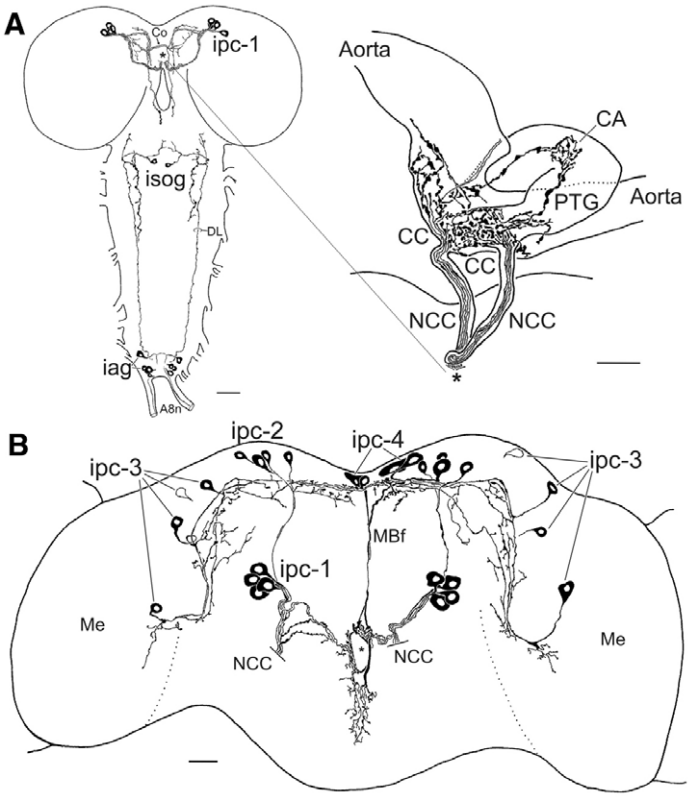

Fig. 5. Camera lucida drawings of identified PAP-stained ITP-immunoreactive neurons in whole mount preparations of larval (L3) CNS and adult brain of *D. melanogaster*. (A) Three larval ITP-immunoreactive neuron types: four pars lateralis NSCs (ipc-1; ITP-immunoreactive protocerebral neuron type), one suboesophageal interneuron (isog), and one to three abdominal (iag) efferent hindgut-innervating neurons; projections of ipc-1 NSC through nervi corporis cardiaci (NCC) to CC with one fibre entering the CA; the ring gland is shown artificially removed (asterisks) and slightly enlarged for clarity. PTG, prothoracic gland. (B) Adult brain showing the distribution of four ipc-1 NSCs, four ipc-2 putative NSCs, four ipc-3-type interneurons innervating a restricted anterior medial lateral neuropile area, and one to two median weakly ITP-immunoreactive ipc-4-type interneurons projecting *via* median bundle fibres (MBf) to SOG neuropiles. Me, medulla. Scale bar 50 μm. After Dircksen et al., with permission (Dircksen et al., 2008).

in total extractable SchgrITP-immunoreactive material, although the bulk of this material was not co-chromatographing with synthetic SchgrITP. However, authentic SchgrITP was clearly identified for the first time in chromatographed haemolymph of fed locusts and occurred in concentrations of about 1 nmol l^{-1}, typical for a circulating neurohormone. At 30 min after feeding, the levels of both peptides were already down again to levels around the detection limit of the assay. This study thus showed, for the first time, SchgrITP and SchgrITPL to be putative hormones in the haemolymph, probably released from the CC of locusts, which led the authors to postulate roles for both peptides in the control of postfeeding osmoregulation. Furthermore, measuring both peptides under stress conditions after chasing the animals for 10 min around their cage revealed that SchgrITPL rather than SchgrITP transiently increased by 1.8-fold. This suggested that humoral SchgrITPL may have additional roles in the regulation of stress metabolism, which may serve as a first preliminary lead towards answering the question of whether ITP and ITPL have further physiological actions. CHHs, for comparison, are known to have at least eight different well established biological effects, some of which are clearly related to stress responses (Böcking et al., 2002; Keller et al., 1999).

The detection of ITP and ITPL in locust CC agrees well with our immunocytochemical localisations of OrcliCHH-immunoreactive material in locust CC and CA (Fig. 3C). The similar distribution for ITPs and ITPLs in the pars lateralis–CC/CA NSC system of the moths points in the same direction. However, contrary to ManseITP immunoreactivity, low amounts of ManseITPL immunoreactivity were only found in cell bodies and parts of the axons of these NSCs but not in CC terminals. Therefore, Dai and colleagues (Dai et al., 2007) concluded that ManseITPL in the brain is not secreted, and its role in this system remains unclear. On the other hand, one can assume that ITPLs are merely released from peripheral sources, i.e. most likely from the homologous and probably all ITPL-containing locust dPSO-associated NSCs, the moths' NS-L1-PVO system, and most of the dipteran LBD neurons. If so, ITPL homologues from these ample sources in the different species could then account for haemolymph concentrations comparable to those observed in the locust. Moreover, based upon these data, an attractive theory on an entirely new competitive type of endocrine control at the level of hindgut ITP receptors, as was put forward earlier by Phillips and colleagues (Phillips et al., 2001), gains a novel dimension. Although shown only *in vitro*, recombinant SchgrITPL itself is not bioactive in the hindgut I_{sc} bioassay but is capable of competing with the highly bioactive SchgrITP for receptor binding which can lead to dramatically diminished bioactivity of SchgrITP (Wang et al., 2000). In blood-feeding insects, distension of the abdominal wall is known to trigger the release of a potent diuretic hormone(s) into the haemolymph followed by increased fluid secretion from MTs (Adams, 1999; Wheelock et al., 1988; Williams et al., 1983; Williams and Beyenbach, 1984). However, ADFs terminating the postprandial diuresis have been proposed to come into play during secretion shut-down, which occurs concomitantly with an increase in cyclic guanosine monophosphate (cGMP) content in *Rhodnius* MTs as the diuresis decreases (Quinlan et al., 1997). Thus, generally assuming that the peripheral ITPL-immunoreactive sensory NSCs have a role as pressure or volume sensory receptors, it would make sense if they mainly released ITPLs. These peptides are inactive themselves but similar enough to compete with the highly bioactive ITPs from brain–CC sources at their supposed and even nearby sites of action on the hindgut to regulate or shut off water uptake. Alternative splicing as a common way of gene regulation would then explain the strikingly similar differential neuronal distributions reviewed here for ITPs and ITPLs in the different insect orders. The functional morphological aspects discussed for the occurrence of these splice forms in the different neuron types would be in accordance with many established physiological findings on hindgut ion and water resorption and the theory of its competitive feedback regulation by different products of the same *itp* gene.

In locusts and moths, another concept for a further possible ITP function has been tested that was mainly based on physiological studies on the role of crab SGCHH in the control of ecdysis as mentioned above. The concept is derived from the observation that SGCHH from gut endocrine cells transiently gives rise to a dramatic increase of CHH haemolymph concentrations leading to water uptake necessary for ecdysis (Chung et al., 1999). However, haemolymph measurements in locusts did not give any clear-cut hint for ecdysis-related changes. Relatively small changes

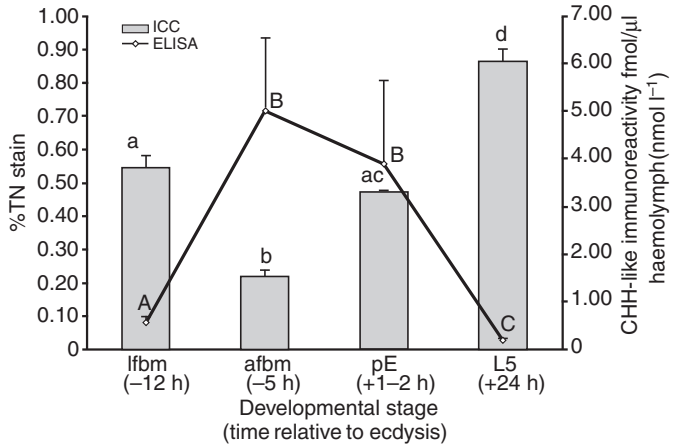

Fig. 6. Changes of CarmaCHH/ManseITP-like peptide immunoreactivity at periods before and after ecdysis in *M. sexta*. Estimated is the abundance of VNC preparations positively stained in transverse nerves (TNs) by immunocytochemistry (ICC, grey columns) and the haemolymph content (rhombi) compared with CHH equivalents in haemolymph samples (non-competitive ELISA with edible crab *Cancer pagurus* SGCHH as a standard). TN immunoreactivity is given as the average of the percentage of preparations in any ganglion showing TN staining. At a proportion of 1.0, all preparations show TN immunoreactivity. All values are represented with s.e.m.; different uppercase letters represent significant differences from other points on the curve at $P<0.05$; differing lowercase letters indicate significant differences from other bars at $P<0.05$. lfbm/afbm, liquid-/air-filled brown mandible stage; pE, post ecdysis stage. Slightly modified from Drexler et al., with permission (Drexler et al., 2007).

compared with the already mentioned feeding-related changes in ITP and ITPL titres were only observed about 5 to 7 days after ecdysis from the fourth to the fifth larval stages of *S. gregaria* (Audsley et al., 2006). Drexler and colleageus (Drexler et al., 2007) set out to measure CHH immunoreactivity and presumed ManseITP-like material (1) densitometrically in immunostained preparations of TN-PVOs and (2) in haemolymph by a non-competitive ELISA (0.25–8 nmol l^{-1} range) before, during and after larval ecdysis from L4 to L5 stages of *M. sexta*. The profiles showed an inverse correlation with the percentage of stained larval TN-PVOs at the same developmental stage around ecdysis whilst the CHH-immunoreactive staining intensities of larval brain type-Ia_2 cell bodies and CC/CA complexes did not differ significantly. Haemolymph levels 5 h before and immediately post-ecdysis were not significantly different from each other but clearly differed from the levels 12 h before and 24 h (L5) after ecdysis (Fig. 6). Thus, elevated haemolymph CHH immunoreactivity levels occurred around the AFBM stage, i.e. when the digestive or moulting fluid has been reabsorbed in the head capsule and tracheae have become filled with air, and immediately following ecdysis, concomitantly with reduced CHH immunoreactivity in TN-PVO. These changes in the profiles of CHH-immunoreactive contents in larval TN-PVOs and haemolymph similar to those observed in crab haemolymph during ecdysis were, therefore, interpreted as indicative of a TN-PVO-released ManseITP-like peptide playing a role in the control of insect ecdysis. Bearing in mind that (1) the CHH-antisera cannot discriminate between the ManseITP/ITPL splice forms and (2) ManseITP and ManseITPL have mutually exclusive distributions in central and peripheral neurons, one would have to interpret that both CHH-immunoreactive peptides have been detected in these haemolymph samples but probably only ManseITPL has been detected in the TN-PVOs. However, none of the other studies on the same moth species (Dai et al., 2007) and on *D. melanogaster* (Dircksen et al., 2008) has found any hint of clearly developmental stage- or ecdysis-related changes in staining intensity of any ITP- or ITPL-immunoreactive neuron.

Possible receptors and second messengers for ITPs and ITPLs

Up to the present date, there are no structural data available for any receptor of CHHs or ITPs/ITPLs; not even assumptions from orphan receptors seem possible. This is perhaps due to the difficulty in obtaining enough peptide material, which would certainly be necessary to deorphanise putative receptors. Early saturation and displacement studies have clearly revealed the existence of specific and high-affinity CHH receptors on hepatopancreas membranes in *C. maenas* and *O. limosus* (Kummer and Keller, 1993). Recently, similarly specific and high affinity binding sites have been found for CarmaCHH on ion transporting gills and hindgut membranes of *C. maenas*, which are coupled to cGMP rather than cAMP as a second messenger (with the exception of the hindgut showing cAMP responses as well). This led the authors to propose an osmoregulatory role for CarmaCHH (Chung and Webster, 2006). Classical binding studies have not yet been undertaken for ITP/ITPL receptors in any insect. It is only known that ion transport-stimulating effects of SchgrITP on the hindgut are probably all mediated by cAMP, i.e. probably *via* a G-protein-coupled receptor (Phillips and Audsley, 1995; Phillips et al., 1998b; Lechleitner et al., 1989a). However, for instance, the known inhibition of ileal acid secretion by ITP is not mimicked by cAMP (Phillips and Audsley, 1995), but there are, at present, no clear hints from physiological studies for cGMP or any other possible second messengers (e.g. Ca^{2+}) being involved in this process. Hitherto only other ADFs such as those from the meal beetle *Tenebrio molitor* (e.g. TenmoADFa) are known to use cGMP as a second messenger for the inhibition of fluid secretion by MTs (Eigenheer et al., 2002; Massaro et al., 2004). When assuming that peptide receptors are as similar as their ligands in crustaceans and insects, which is to date known only for some serotonin and dopamine receptors (Clark et al., 2004; Clark and Baro, 2006; Spitzer et al., 2008), one would probably have to expect G-protein-coupled receptors but cannot exclude membrane-bound guanylyl cyclases as mediators of some of the known physiological effects of ITP isoforms.

Conclusions and perspectives

Insect ion transport peptides and crustacean hyperglycaemic hormones are derived from alternative splicing of *itp* and *chh* genes. They are differentially expressed in the CNS and the PNS of these arthropods. Four types of neuron produce ITP or ITPL: (1) typical usually pars lateralis NSCs in the protocerebrum with putative release sites in the CC and CA (for ITP and possibly ITPL), which are similar, if not homologous, to crustacean CHH-producing X-organ sinus gland neurons in the medulla terminalis of the eyestalk, which is considered to be a lateral brain neuropile; (2) interneurons which may connect NSCs to other neuron types; (3) efferent neurons, e.g. those hitherto only found in terminal AGs of *D. melanogaster*, which innervate the hindgut directly and produce DromeITP; and (4) peripheral putative sensory NSCs probably producing ITPLs only. Furthermore, the localisation of CHH/ITP-immunoreactive material distinctly around the juvenile hormone-producing endocrine cells but only little in cortical neurohaemal areas of the CA suggests that the intrinsic CA cells are a possible novel target organ for ITPs. This is particularly striking because this projection pattern of pars lateralis

ITP-immunoreactive NSCs has now been described for all hitherto investigated insects with the possible exception of the stick insect. When considering the multitude of pleiotropic functions of SGCHHs (Böcking et al., 2002; Keller et al., 1999), more functions may well have to be expected for the similar humoral ITPs. The available results have shown a remarkable conservation and similar differential distributions of ITP and ITPL neurons in orthopteran, lepidopteran and dipteran insects. This actually very much resembles the situation in crustaceans. Despite the fact that CHH-like peptides have not yet been found in typical interneurons, it is tempting to speculate that the SGCHH NSCs of the eyestalk medulla terminalis X-organ sinus gland system as part of the lateral brain proper, and the peripheral intrinsic pericardial organ POCHH NSCs compared with the ITP(/ITPL) NSCs of the brain pars lateralis and the TN-associated NSCs intrinsic to the neurohaemal PSO/PVO and the LBD neurons are two principally homologous neuron types with homologous signalling molecules derived from homologous genes. Thus, these neuron types with similarly expressed genes in insects and crustaceans may have arisen from a common phylogenetic ancestor for the two arthropod groups. In this context, an important next step will definitely be the identification and localisation of ITP receptors in suspected target organs such as the hindgut and the CA. This will spawn novel research on the fascinating integrative aspects of a highly efficient insect kidney under the control of several probably phylogenetically old diuretic and antidiuretic neurohormonal systems. Approaches to localise identified ITP receptors throughout the whole bodies of insects will, furthermore, provide the first hints of the existence of probably many other and possibly homologous target organs, receptors and functions common to ITP and CHH isoforms in both arthropod groups. This aspect can, at present, only be assumed from the manifold physiological studies done on crustaceans.

List of abbreviations

ADF	antidiuretic factor
AG	abdominal ganglia
CA	corpora allata
CC	corpora cardiaca
CHH	crustacean hyperglycaemic hormone
CNS	central nervous system
CTSH	chloride transport-stimulating hormone
dPSO	dorsal perisympathetic organ
iag	ITP-immunoreactive abdominal neuron
ipc	ITP-immunoreactive protocerebral neuron
isog	ITP-immunoreactive suboesophageal neuron
ITP	ion transport peptide
LBD	lateral bipolar dendrite
LN	link nerve
MT	Malpighian tubules
NSC	neurosecretory cell
PNS	peripheral nervous system
PVO	perivisceral organ
SOG	suboesophageal ganglia
TG	thoracic ganglia
TN	transverse nerve
VNC	ventral nerve cord

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