The Light Green Cells of *Lymnaea*: A neuroendocrine model system for stimulus-induced expression of multiple peptide genes in a single cell type

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Abstract. We review recent experiments showing that the cerebral neuroendocrine Light Green Cells (LGCs) of the freshwater snail, *Lymnaea stagnalis*, express a family of distinct though related molluscan insulin-related peptide (MIP) genes. The LGCs are involved in the regulation of a wide range of interrelated life processes associated with growth, (energy) metabolism and reproduction. We consider the mechanism of generation of diversity among MIPs, and present evidence that conditions with distinct effects on growth, metabolism and reproduction also can induce distinct patterns of expression of the MIP and schistosomin genes. The stimulus-dependent expression of multiple neuropeptide genes enormously increases the adaptive potential of a peptidergic neuron. We suggest that this contributes significantly to the information-handling capacity of the brain.
Key words. Molluscan insulin-related peptides; schistosomin; neuropeptide gene family; generation of neuropeptide diversity; stimulus-dependent expression; information-handling capacity; evolution.

Introduction

Neuropeptides form a class of information-rich signal molecules, and a current focus of interest concerns the significance of the great diversity of these peptides for the information processing activities of the brain. The molluscan central nervous system (CNS) is an advantageous model system for the investigation of issues related to this problem. The molluscan CNS consists of a relatively small number ($\sim$ 15,000) of large identifiable neurons, which are grouped in a few ganglia and which, in addition, express extensive peptide heterogeneity. In the freshwater gastropod, *Lymnaea stagnalis*, more than 50 neuropeptides have been structurally identified in recent years by the application of recombinant DNA methodology and protein chemistry. Many of these peptides are produced by neurons with a known function, whose basic characteristics are being studied using an interdisciplinary approach.

The biosynthesis of neuropeptides occurs by standard synthetic mechanisms. Like many other eukaryotic genes, molluscan neuropeptide genes are split genes. The intervening sequences are spliced out of the primary transcript to form mature RNA, which is then translated in the endoplasmic reticulum to yield the preprohormone. After removal of the signal peptide the prohormone is packaged into secretory granules in the Golgi apparatus, where it is processed to generate the bioactive peptides, which are transported within the granules, and then released. As listed in table 1, in *Lymnaea* most of the prohormone and peptide diversity observed to date is mediated by families of related genes encoding prohormone subtypes, and by alternative splicing of exons contained within a single neuropeptide gene. Alternative mechanisms, such as intragenic duplications of peptide coding regions, and alternative processing and post-translational modifications, also have roles in generating peptide heterogeneity.

Gene duplication produces gene families that code for prohormone subtypes. It allows alterations within the entire prohormone coding sequence, thereby creating diversity in the various peptide domains of the prohormone. The members of a gene family are expressed in a cell- and tissue-specific as well as a developmentally regulated fashion. Alternative splicing also produces prohormone subtypes, but it only allows specific portions of the prohormone to be varied. Alternative splicing is also used in cell- and tissue-specific as well as developmental expression of subtypes of prohormones. However, having a second gene (or genes) to determine the cell specificity of prohormone subtype expression is no more and no less economical than having two distinct prohormone genes to encode subtype heterogeneity.

The use of related genes to encode prohormone subtype heterogeneity appears to offer the greatest flexibility. The use of distinct genes suggests that transcriptional control of gene expression has an important role in prohormone

Generation of prohormone and neuropeptide diversity in *Lymnaea**

Prohormone family	Diversity of prohormones and peptides generated by	References
SIS	One gene	(12)
APGWamide	One gene	(12)
Conopressin	One gene (?)	(11, 19)
CDCH	Gene family	(14)
MIP	Gene family	(14)
FMRFamide	Alternative RNA splicing	(12, 22)
VD_1/RPD_2	Alternative RNA splicing	(12)
LYC	Alternative RNA splicing**	(12)
FMRFamide	Prohormone consisting of multiple copies of peptides	(22)
CDCH	Alternative processing	(12, 14)

*Abbreviations: CDCH, caudodorsal cell hormone; FMRFamide, Phe-Met-Arg-Phe-amide; LYC, light yellow cell; MIP, molluscan insulin-related peptide; VD_1/RPD_2, visceral-dorsal ganglion cell no. 1/right parietal dorsal ganglion cell no. 2. **Splicing of noncoding exons.

subtype heterogeneity. This may include developmental, as well as tissue- and cell-specific transcription, the use of cellular factors to alter transcriptional levels of various subtypes of prohormone independently, and also the ability to alter specifically the levels of prohormone subtypes present within a given cell. The various inputs received by a cell can regulate the levels of intracellular second messengers and these levels can directly or indirectly control subsequent gene expression. Thus, depending on the input signals received, specific expression of a prohormone subtype or subtypes can be altered.

A promising experimental model for the study of the expression of multiple (related yet distinct) genes in one type of neuron is offered by *Lymnaea*, where several members of a gene family encoding the different yet related molluscan insulin-related peptides (MIPs) are expressed in the neuroendocrine Light Green Cells (LGCs). Here, we will review recent experiments concerning the organization, neuronal expression, and evolution of the MIP genes, as well as the biosynthesis of the bioactive peptides derived from the prohormones. We will, in addition, discuss a novel mechanism, not described hitherto, for the generation of peptide diversity among members of a neuropeptide family, and examine the stimulus-induced differential expression of the MIP genes in the LGCs. We suggest that similar mechanisms are operative in peptidergic neurons of the vertebrates. In the next section, we begin by presenting an anatomical description of the LGC system and the various life processes of *Lymnaea* that are regulated by the LGCs.

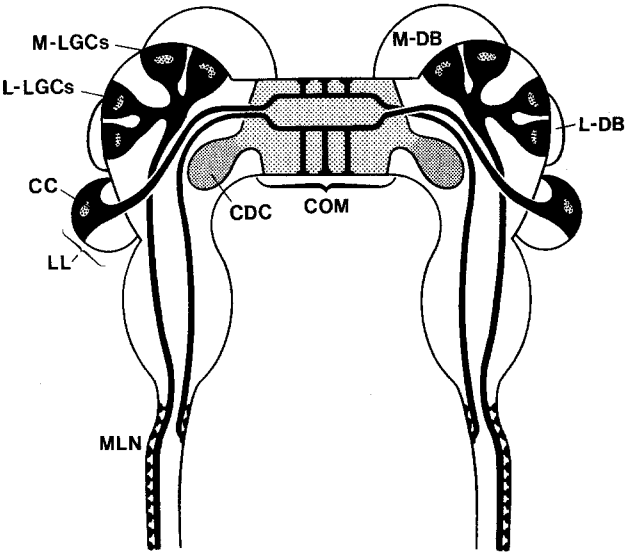

Figure 1. Location and anatomical organization of the LGC system in the cerebral ganglia of *L. stagnalis*, based on Lucifer Yellow fillings[2] and light and electron microscopy[17,23]. Notice the intricate axonal topology of the canopy cells (CC) in the lateral lobes (LL), which strongly suggests that the CC control the activities of the other LGCs and of the female gonadotropic centers, the caudodorsal cells (CDC) and dorsal bodies (DB) (see text). M-LGC and L-LGC, medio- and laterodorsal groups of LGCs, resp.; MLN, median lip nerve, the neurohemal area of the LGCs; COM, cerebral commissure, the neurohemal area of the CDCs; M-DB and L-DB, medio and laterodorsal bodies, resp.

Anatomy and functional role of the LGCs

The LGCs are giant neurons (diameter 90 μm), which are located as two clusters in each cerebral ganglion in *Lymnaea*, one in the medio- and one in the laterodorsal part (fig. 1)[17,31]. Together, the four clusters comprise ~ 150 cells. They use the periphery of the long median lip nerves as their neurohemal area. The LGCs show all the characteristics typical of protein-synthesizing cells with elaborate stacks of endoplasmic reticulum, many active Golgi complexes and numerous neurosecretory granules and show the exocytosis profiles typical of peptidergic (neuroendocrine) neurons.

The experimental proof that the LGCs are involved in the control of growth of *Lymnaea* comes from classical endocrinological extirpation and implantation experiments[7]. Cauterization of the LGCs of rapidly growing juvenile snails results in a marked retardation of body growth, which can be restored by implantation of cerebral ganglia containing the LGCs. Recent experiments have shown that the LGCs stimulate growth of the soft body parts by stimulating cell multiplication[9]. The LGCs also affect various aspects of metabolism which are related to growth[9,13]. Thus, factors released from the LGCs keep glycogen stores low, and stimulate the activity of ornithine decarboxylase, an enzyme which shows high levels of activity in growing animal tissues. The LGCs further stimulate various processes of shell growth: formation of the periostracum, the proteinaceous component of the shell, as well as calcium and bicarbonate incorporation into the shell edge, and the maintenance of high concentrations of a calcium-binding protein, important for cellular calcium transport in the mantle edge.

In *Lymnaea*, a second center involved in growth control is located in the lateral lobes (fig. 1). Cauterization of the lobes results in giant growth, whereas reimplantation of cerebral ganglia with lateral lobes restores normal growth[8]. The effects of the lateral lobes are mediated via changes in the activities of the LGCs. An interesting observation is that in each lobe an ectopic LGC, termed the canopy cell, is located. The axons of both canopy cells show an intricate branching pattern; they cross over to the opposite ganglion and run very close to axons coming from the clusters of LGCs in the cerebral ganglia[2,23]. In the commissure they give off collaterals that run to the female gonadotropic centers, the caudodorsal cells and the dorsal bodies (fig. 1). This suggests that the canopy cells are specialized LGCs that transmit regulatory stimuli to the LGC clusters in the cerebral ganglia as well as to the female gonadotropic centers (see below).

The peptide messengers produced by the LGCs have been identified using the methodologies of both molecular biology and peptide chemistry. The LGCs produce at least four different MIPs, each of which is thought to control a distinct aspect of growth and associated

processes in *Lymnaea*[14,26]. The MIPs possess the basic three-dimensional configuration, with disulphide bridges, a hydrophobic core, α-helices, and sharp turns of the peptide chains, typical of other members of the insulin superfamily, i. e., the insulins, insulin growth factors (IGFs), and relaxins of the vertebrates[5,23], and the bombyxins of the insects[18]. The organization, the expression, and the evolutionary aspects of the MIPs and the gene family encoding them are reviewed in the sections that follow. The dual effects of schistosomin on growth and reproduction will be discussed in a later section.

The organization of the MIP gene family

Using a plus/minus screening strategy[26], four LGC-specific cDNA clones encoding the precursors of MIPs I, II, III and V were identified. The corresponding MIP I, II, III and V genes were isolated with the help of radioactive probes made from the MIP cDNAs and from synthetic gene-specific oligonucleotide sequences derived from the cDNAs. Two more genes, the MIP IV and VI genes, were identified in the course of the genomic analysis (see Geraerts et al.[14]). Highly interesting features of the organization of the MIP genes were revealed when the MIP genes were compared with the vertebrate insulin genes, e.g., the human insulin gene (fig. 2). All MIP genes show the overall structure typical of the insulin genes, with three exons interspaced with two introns. The exons code for similar domains of the precursors in both the MIP genes and insulin genes. The positions of the introns are also conserved, one within the sequence coding for the mRNA leader region and the other interrupting the C peptide coding region. The six MIP genes are present in the genome as three couples of closely linked genes, which are transcribed in the same direction. They represent all the homologous members of the MIP gene family in the *Lymnaea* genome, as indicated by genomic Southern blotting and restriction analysis of separate genomic clones. The family may, however, include additional members with undetectably low homology to the identified MIP genes. The genomic organization of the MIP gene family differs markedly from that of the bombyxin gene family[18]. Bombyxin genes do not contain introns and are arranged as transcription units of paired genes with opposite orientation.

A TATA box is present at the expected position (approx. −25) in the 5′-region of the MIP I, II, III, and V genes. By contrast, in both the MIP IV and VI genes, a putative TATA box is located further upstream (at position −178). Two transcripts, which differ in their leader sequence by 83 nucleotides, can be generated from the MIP I gene by alternative splicing. The stretches of the 5′-flanking regions near the transcription initiation sites of the MIP I and II genes are quite similar, but sequence divergence becomes gradually stronger further upstream. The 5′-flanking regions of the MIP II and V genes are also very similar, except for a few interspersed stretches of pronounced sequence divergence. By contrast, the 5′-flanking region of the MIP III gene has no sequence resemblance with those of other MIP genes. The upstream regions, the signal peptide, and the first amino acids of the B chain in the MIP IV and VI genes are very similar to each other. Also, the upstream regions do not resemble those of other MIP genes. The MIP VI gene is probably a pseudogene, because an insertion of 7 nucleotides in exon 2 causes a shift of the reading frame resulting in a stop codon at the end of exon 2. For reasons discussed below, the MIP IV gene might be a pseudogene as well.

Biosynthesis, structural characteristics and central expression of the MIPs and their precursors

The MIP precursors encoded by the completely characterized MIP I, II, III and V genes represent preproinsulin-related proteins containing a signal sequence, A and B chains, and a connecting C peptide, as well as dibasic amino acid processing sites for the generation of MIPs and C peptides (fig. 3). Pulse-label and pulse-chase analysis of newly synthesized proteins and peptides in the LGC system in vitro showed that putative MIP precursors were present in the LGC somata after a 20-min pulse with radioactive cysteine. The synthesis and subsequent conversion of the proMIPs to end products is probably

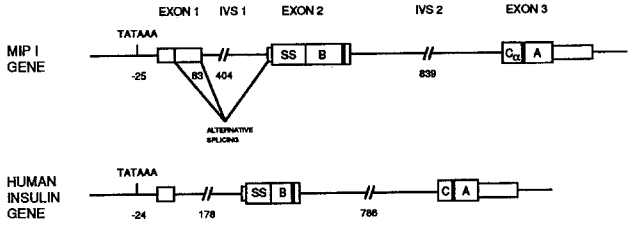

Figure 2. Schematic representation of the MIP I gene and the human insulin gene. The MIP genes II–VI have a similar organization. Indicated are the exons and the intervening sequences (IVSs). SS, signal sequence; B, B chain; A, A chain; Cα, Cα peptide; C, C peptide. Goldberg-Hogness box, TATAAA. Numbers refer to nucleotide length. The alternative splicing in exon 1 of the MIP I gene is also indicated.

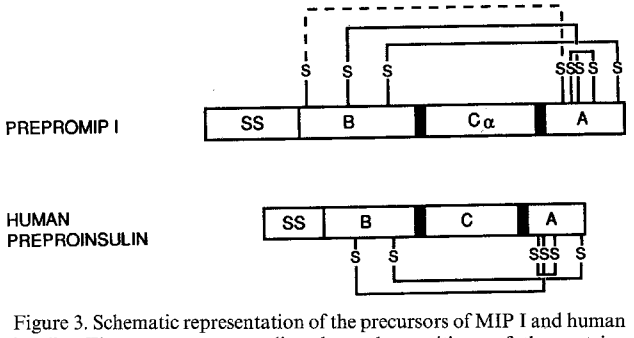

Figure 3. Schematic representation of the precursors of MIP I and human insulin. The precursors are aligned on the positions of the cysteine residues involved in the interchain disulphide bridges in the B chain domains. SS, signal sequence; B, B chain; A, A chain; Cα, Cα peptide; C, C peptide; S, position of cysteine residue. The extra putative disulphide bridge in MIP is indicated by a dotted line, others by solid lines. Vertical black bars indicate proteolytic cleavage sites.

confined to the LGC somata (and perhaps the proximal parts of the LGC axons, which were not studied in these experiments). The MIPs and C peptides were transported to the LGC axon terminals in the median lip nerves, where they appeared between 1.5 h and 6 h after the pulse period. Sequencing of the material purified from the median lip nerves showed that the MIPs I, II, III and V were indeed present, in addition to the Cα peptide. Thus, the data show that, overall, the processing of preproMIPs is very similar to that of preproinsulin in the β cells of pancreatic islets.

Close inspection of the cDNA and peptide data reveals a number of differences between the various MIPs. The A and B chains of MIPs I, II and V appear to be terminally blocked (pyroglutamate), and the B chain N-terminus of MIP III is two amino acids longer than would be predicted by the gene analysis. Moreover, the two C-terminal amino acids of all B chains are post-translationally removed. These data indicate that preproMIPs are processed to form mature 2-chain MIP molecules which are stored in the LGC axon terminals, together with the various C peptides. Comparison of MIPs I, II, III and V shows that they are homologous. Only about 45%–75% of the amino acid residues are identical among them throughout the A and B chains (fig. 4). However, all MIPs share the amino acids that are important in adopting the basic insulin configuration. This is apparent when MIPs are compared with other members of the insulin superfamily (fig. 4). In the A and B chains of the MIPs, cysteines are present at positions typical for the insulin superfamily, which suggests that the three characteristic disulphide bridges in the MIP molecules have been conserved. In addition, the important hydrophobic core residues of the globular insulin structure are either conserved as identical residues, or are replaced by residues with an equally hydrophobic character. The only exception is arginine at B15 in MIPs II and V. Also, α-helices, which are present in the A and B chains of vertebrate insulins, can be predicted for the MIPs.

Although the MIPs are 2-chain peptides that will adopt the insulin core structure, they cannot be considered to be genuine insulin molecules. MIPs exhibit a strong divergence of the surface residues which are important in many functions of the vertebrate insulin molecules, such as receptor binding, solubility of the molecule, processing of the precursor, monomer and dimer interactions, as

A Chains

		-4	-3	-2	-1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Insulin(-related)																										
MIP I	Lymnaea	Q	G	T	T	N	I	V	C	E	C	C	M	K	P	C	T	L	S	E	L	R	Q	Y	C	P
MIP II	Lymnaea	Q	R	T	T	N	L	V	C	E	C	C	F	N	Y	C	T	P	D	V	V	R	K	Y	C	Y
MIP III	Lymnaea	E	S	R	P	S	I	V	C	E	C	C	F	N	Q	C	T	V	Q	E	L	L	A	Y	C	-
MIP V	Lymnaea	Q	R	T	T	N	L	V	C	E	C	C	Y	N	V	C	T	V	D	V	F	Y	E	Y	C	Y
bombyxin II	Bombyx	-	-	-	-	G	I	V	D	E	C	C	L	R	P	C	S	V	D	V	L	L	S	Y	C	-
Insulin (-related)	Sponge [9]	-	-	-	-	-	I	V	Q	Q	C	T	S	G	I	C	S	L	Y	Q	-	E	N	Y	C	N
IGF I	Human	-	-	-	-	G	I	V	D	E	C	C	F	R	S	C	D	L	R	R	L	E	M	Y	C	A ...
IGF II	Human	-	-	-	-	G	I	V	E	E	C	C	F	R	S	C	D	L	A	L	L	E	T	Y	C	A ...
Relaxin	Human	R	P	Y	V	A	L	F	E	K	C	C	L	I	G	C	T	K	R	S	L	A	K	Y	C	-
Relaxin	whale [10]	-	-	R	M	T	L	S	E	K	C	C	Q	V	G	C	I	R	K	D	I	A	R	L	C	-
Insulin	Hagfish	-	-	-	-	G	I	V	E	Q	C	C	H	K	R	C	S	I	Y	N	L	Q	N	Y	C	N
Insulin	Guinea pig	-	-	-	-	G	I	V	D	Q	C	C	T	G	T	C	T	R	H	Q	L	Q	S	Y	C	N
Insulin	Rat I	-	-	-	-	G	I	V	D	Q	C	C	T	S	I	C	S	L	Y	Q	L	E	N	Y	C	N
Insulin	Human	-	-	-	-	G	I	V	E	Q	C	C	T	S	I	C	S	L	Y	Q	L	E	N	Y	C	N

B Chains

		-10	-9	-8	-7	-6	-5	-4	-3	-2	-1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
Insulin(-related)																																										
MIP I	Lymnaea	Q	F	S	A	C	N	I	N	D	R	P	H	R	R	G	V	C	G	S	A	L	A	D	L	V	D	F	A	-	C	S	S	S	N	Q	P	A	M	V	-	-	-
MIP II	Lymnaea	Q	-	S	S	C	S	L	S	S	R	P	H	P	R	G	I	C	G	S	N	L	A	G	F	R	A	F	I	-	C	S	N	Q	N	S	P	S	M	V	-	-	-
MIP III	Lymnaea	Q	-	H	T	C	S	I	L	S	R	P	H	P	R	G	L	C	G	S	T	L	A	N	M	V	Q	W	L	-	C	S	T	Y	T	T	S	S	K	V	-	-	-
MIP V	Lymnaea	Q	F	S	A	C	S	F	S	S	R	P	H	P	R	G	I	C	G	S	D	L	A	D	L	R	A	F	I	-	C	S	R	R	N	Q	P	A	M	V	-	-	-
Bombyxin II	Bombyx	-	-	-	-	-	-	-	E	N	P	N	A	V	H	T	T	C	G	R	H	L	A	R	T	L	A	D	L	-	C	W	E	A	G	V	D	-	-	-	-	-	-
Insulin (-related)	Sponge	-	-	-	-	-	-	-	-	-	-	F	V	N	Q	H	L	C	G	S	H	L	V	E	A	L	Y	I	L	V	C	G	E	R	G	F	F	Y	T	P	M	S	-
IGF I	Human	-	-	-	-	-	-	-	-	-	-	-	G	P	E	T	L	C	G	A	E	L	V	D	A	L	Q	F	V	-	C	G	D	R	G	F	Y	F	N	K	P	T	G
IGF II	Human	-	-	-	-	-	-	-	-	A	Y	R	P	S	E	T	L	C	G	G	E	L	V	D	T	L	Q	F	V	-	C	G	D	R	G	F	Y	F	S	R	P	A	S
Relaxin	Human	-	-	-	-	-	-	-	K	W	K	D	D	V	I	K	L	C	G	R	E	L	V	R	A	Q	I	A	I	-	C	G	M	S	T	W	S	K	R	S	L	-	-
Relaxin	Whale	-	-	-	-	-	-	-	-	S	T	N	D	L	I	K	A	C	G	R	E	L	V	R	L	W	V	E	I	-	C	G	S	V	S	W	G	R	T	A	L	-	-
Insulin	Hagfish	-	-	-	-	-	-	-	-	-	-	R	T	T	G	H	L	C	G	K	D	L	V	N	A	L	T	I	A	-	C	G	V	R	G	F	F	Y	D	P	T	K	M
Insulin	Guinea pig	-	-	-	-	-	-	-	-	-	-	F	V	S	R	H	L	C	G	S	N	L	V	E	T	L	T	S	V	-	C	Q	D	D	G	F	F	Y	I	P	K	D	-
Insulin	Rat I	-	-	-	-	-	-	-	-	-	-	F	V	K	N	H	L	C	G	P	H	L	V	E	A	L	Y	L	V	-	C	G	E	R	G	F	F	Y	T	P	K	S	-
Insulin	Human	-	-	-	-	-	-	-	-	-	-	F	V	N	Q	H	L	C	G	S	H	L	V	E	A	L	Y	L	V	-	C	G	E	R	G	F	F	Y	T	P	K	T	-

Figure 4. Amino acid sequence comparison of MIPs I, II, III and V with those of other insulin(-related) peptides. The sequence similarity in the A and B chains of the MIPs ranges from 45%–75%. The sequence similarity of the MIPs to those of bombyxin II, sponge insulin-related peptide [25], and vertebrate insulins, relaxins, and IGFs is 20–40%. The sequence similarity among insulins and related peptides from vertebrates is 55–95%. In the MIP A and B chains cysteines are present at positions A6, A7, A11, A20, B7 and B19, an arrangement which is typical for members of the insulin superfamily. Two additional cysteines are present at positions A4 and B6. Like the insulins, MIPs have a glycine at B8, but lack glycines at B20 and B23, which are present in most insulins and introduce a sharp turn in the B chain. Most residues of the hydrophobic core of insulin are conserved as hydrophobic in the MIPs, namely A2, A11, A16, A20, B11, B15 and B19, except for Arg at B15 in MIPs II and V. Other hydrophobic residues are identical or conserved: A3, A19, B6, B12 and B18. Residues A1–A5, A8, and B23–B26, which interact with the insulin receptor, are diverged among MIPs. Number 1 designates the first residues of the A and B chains of human insulin.

well as hexamer formation. A striking example is the almost complete divergence of the amino acid residues involved in receptor binding, including residues at A1–A5, A8, and B23–26. Thus, it is unlikely that the MIP receptor is of the vertebrate type. Interestingly, these putative receptor binding residues are divergent among MIPs, which suggests either that each MIP binds a different receptor, or alternatively, that various MIPs bind the same receptor with different affinities. Thus, each MIP may fulfill a different function in the control of growth and associated processes in *Lymnaea*. In addition to these differences, MIPs possess features that classify them into a distinct group of the insulin superfamily. Both the A and B chains are N-terminally extended, and the B chain extension contains an extra cysteine at B-6 that may form a third interchain disulphide bridge with an extra cysteine at A4. Because of these properties, the MIPs are the most completely folded molecules of the insulin superfamily.

Expression of the MIP I, II, III and V genes in adult animals is entirely restricted to the LGCs, as could be assessed by various methods. Northern blotting experiments showed that MIP I, II, III and V transcripts are only present in the cerebral ganglia of the CNS, while no MIP IV and VI transcripts could be identified. Because the MIP VI gene is probably a pseudogene, this is not surprising. For the same reason the MIP IV transcript may not have been found. Alternatively, however, MIP IV gene transcription may take place under conditions that are as yet unknown. The MIP I, II and V specific transcripts each had a length of 650–700 nucleotides. Two MIP III specific transcripts of 800 and 1150 nucleotides have been found; these are probably generated by the use of two distinct termination signals in the 3′-region of the MIP III gene.

Hybridization histochemistry showed that the MIP I, II, III and V genes are expressed in the LGCs and in the canopy cells of the lateral lobes. No other cell type of the CNS showed a positive signal. Unexpectedly, MIP transcripts were also detected in LGC axons. The significance of this phenomenon is not well understood, and further experiments to investigate it are in progress. The immunohistochemistry was in agreement with the in situ hybridization studies and showed immunostaining only of LGCs and canopy cells. Furthermore, it revealed a complex axonal topology of the LGCs and canopy cells that confirms the conclusions from dye injections and from the ultrastructural studies in all details (fig. 1).

To determine the intracellular localization of the (pro)MIPs more precisely, immunogold electron microscopy was performed. Immunogold labeling in both the LGCs and canopy cells was observed above neurosecretory granules budding off from the Golgi apparatus, above mature granules in both the cell bodies and axon terminals, and over exocytosis profiles in LGC axon terminals. This suggests that proMIPs are packaged into granules in the Golgi apparatus and transported to the axon endings, where the peptides derived from the precursors are released. There is endocrinological evidence that the lateral lobes are involved in the control of the synthesis and release activities of the LGCs and of female gonadotropic centers in the cerebral ganglia[8]. Therefore, the canopy cells probably are specialized LGCs that transmit regulatory stimuli to both the LGC clusters and the female gonadotropic centers (fig. 1) of the cerebral ganglia.

Stimulus-dependent differential expression of the MIP genes in the LGCs

Since each MIP may have a different function, we reasoned that physiological conditions with different effects on growth and associated processes in *L. stagnalis* might induce a differential pattern of expression of the MIP genes in the LGCs. We first investigated the effects of starvation on MIP transcript levels. During starvation, growth is arrested, glycogen stores are considerably depleted, and reproductive activities stop. Northern blot analysis of MIP II and III transcripts in the LGCs showed that during starvation the MIP II transcript had disappeared completely, while the level of the MIP III transcript was reduced. Furthermore, the length of the MIP III transcript was reduced in starved animals, indicating changes in mRNA stability and/or translation efficiency. In a second series of experiments two treatments were studied: 1) the effects of extirpation of the lateral lobes, a treatment which causes giant growth, a depletion of glycogen stores, and a decrease of reproductive activities; and 2) the effects of a carbohydrate-rich diet, Bemax, which results in an arresting of growth, enormously increased glycogen stores, and a stimulation of reproduction. After removal of the lateral lobes the transcript levels of all four MIPs (I, II, III and V) increased, although not to the same degree. Changing the diet from lettuce to Bemax was followed by a decrease in transcript levels of MIPs II and III. The results to date do indeed suggest a stimulus-dependent differential pattern of expression of the MIP genes in the LGCs.

Electrical activity and release of MIPs

Data concerning the electrophysiological characteristics of the LGC system are restricted to in vitro experiments on LGCs in the isolated CNS or on dissociated LGCs in primary culture. These in vitro studies have shown that the LGC system can act as a site of integration of diverse information. Among the first messengers involved in the regulation of LGC activities are several peptides and amino acids, as well as dopamine and glucose. Only the dopamine and the glucose responses have been studied in some detail. The LGCs of *Lymnaea* display a complicated response to dopamine[29]. Initially, the LGCs are rendered inexcitable. This is caused by a hyperpolarization, due to the opening of K-channels, and by a concomittant reduction of the voltage-dependent Ca-current. The ef-

fect on the K-channels is mediated via a D-2 like receptor. This initial phase of the response lasts from one to a few minutes and is followed by a slow rise in excitability, during which the cells gradually depolarize. The effect is mediated through a D-1 like receptor. Dopamine showed no effect on the intracellular cAMP levels of the LGCs, therefore it is unlikely that the responses are mediated through inhibition or stimulation of adenylate cyclase activity, as is the case in the classical vertebrate D-2 and D-1 receptors. However, an increase in the intracellular levels of cAMP using non-hydrolyzable analogs, such as 8-CPT-cAMP, prevented the inhibitory response and mimicked the stimulatory one. Therefore, the role of cAMP in the regulation of the responses to dopamine remains as yet unresolved.

An interesting finding is that physiological concentrations of glucose (0.1–10 mM) applied in vitro can induce long-lasting discharges of action potentials in the LGCs (fig. 5)[20]. The glucose response is evoked by D-glucose, but not by L-glucose or other hexoses, nor by pentoses or disaccharides. The glucose response depends on the presence of extracellular Na^+ and is blocked by phlorizin, suggesting that the response is due to activation of an electrogenic Na^+-coupled glucose transporter. Thus, blood glucose may play an important role as an excitatory first messenger. This creates an interesting parallel with the regulation of the electrical activities of the β-cells in the pancreas of vertebrates[24]. There is strong evidence that the regulation of LGC excitability involves cAMP acting on Ca-channels[21]. When the level of cAMP is experimentally increased, inexcitable cells turn into excitable ones, and cells that had previously displayed enhanced excitability initiate a long-lasting spiking activity. Application of 8-CPT-cAMP, IBMX and forskolin cause enhancement of the Ca-current, which can be mimicked by intracellular application of the catalytic subunit of cAMP-dependent kinase. This suggests that the effect is brought about by phosphorylation of Ca-channels or associated proteins. Therefore, it is thought that an increase in the intracellular levels of cAMP and calcium is necessary for release of MIPs from the LGCs. This is entirely corroborated by the results of in vitro experiments employing LGC systems, prelabeled with radioactive cysteine and treated with cAMP (K. W. Li, unpublished results). Application of cAMP clearly stimulates release of MIPs above background levels. Other compounds that induce spiking activity in the LGCs, such as glucose and 4-aminopyridine, are also capable of stimulating the release of MIPs.

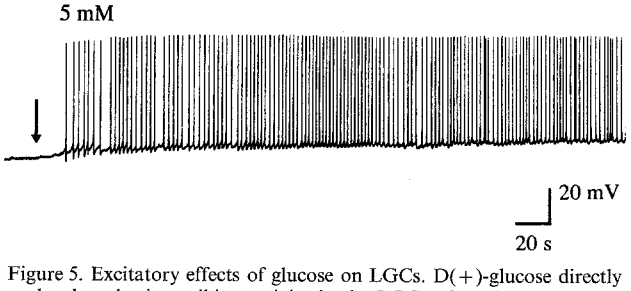

Figure 5. Excitatory effects of glucose on LGCs. D(+)-glucose directly evokes long-lasting spiking activity in the LGCs of cerebral ganglia in vitro. D-glucose was applied at the arrow. The effect is not seen with L-glucose or with other sugars. Courtesy, Dr Kits.

Evolutionary perspective

The insulin superfamily of regulatory peptides signals essential steps in growth, development, reproduction, and metabolism in both vertebrates and invertebrates. The members of the insulin superfamily are quite divergent, structurally and functionally. In vertebrates, the 2-chain insulins are released by the pancreas and function as metabolic hormones, whereas the single-chain IGFs and the 2-chain relaxins are produced by different tissues, bind different receptors, and serve other functions. IGFs are important growth regulators, and relaxins have a role in reproduction. In molluscs, MIPs are involved in the control of growth and associated metabolic processes. The members of the insulin superfamily can be subdivided into distinct groups of related peptides, and their phylogenetic relationships can be tentatively proposed as shown in figure 6. The functional and structural diversity of the members of the insulin superfamily raises many important questions and urges the development of new concepts concerning the evolution, phylogenetic distribution, and function of these peptides. Some of these issues will be discussed below.

All members of the insulin superfamily share the basic insulin globular configuration, indicating that an early genesis of this information-rich structure has been an important step in the evolution of the superfamily. Because this basic insulin motif is found in vertebrates, insects and molluscs, it must have been present in the common ancestors of these organisms, the Archaemetazoa, which date back as far as 6.10^8 years. The insulin-related peptide of sponges exhibits very high amino acid sequence similarity with the vertebrate insulins (cf. fig. 4). However, it is worrying that the intrachain disulphide bridge in the A chain is absent, and the positions of the other cysteine residues are atypical, owing to a deletion in the A chain and an insertion in the B chain. These modifications may have dramatic consequences for the three dimensional structure. In the MIPs, a fourth disulphide bridge occurs. This probably indicates a relatively recent development towards a more complex insulin core structure.

The studies on the MIP genes provide the first indication that the ancestral insulin-related peptides of the Archaemetazoa were encoded by genes that already possessed the structural organization of the insulin gene. They furthermore suggest that these ancestral peptides were proteolytically cleaved from a prohormone in much the same way as the insulins and MIPs. Thus, the IGF genes, which have a modified intron-exon organization[27,28] and code for single-chained peptides, and the

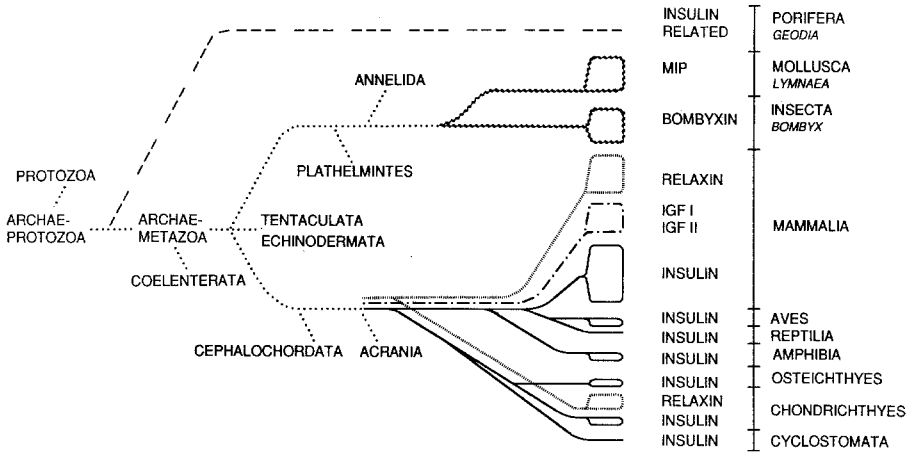

Figure 6. Tentative scheme of the phylogenetic relationships of the animal phyla and the known members of the insulin superfamily. Dotted lines, hypothetic phylogeny. Dashed line, insulin-related peptide of sponges. Wavy lines, insulin-related peptides of insects and molluscs. Cross-hatched lines, relaxins of vertebrates. Dashed/dotted lines, IGFs of vertebrates. Solid lines, insulins of vertebrates.

bombyxin genes, which lack introns altogether [18], probably represent more recently evolved modifications of the ancestral insulin genes.

Interestingly, in the molluscs and insects the insulin-related peptides are produced by neuroendocrine cells in the CNS. The claim that insulin is produced by neurons in the CNS of vertebrates is controversial. However, in view of the growing evidence that pancreatic islet cells share a number of features with neuroendocrine cells [1], it seems possible that in the Archaemetazoa the evolution of the insulin superfamily may have been within primitive neuroendocrine cells, probably associated with the digestive system [27, 28]. In *Lymnaea* and other invertebrates, there is evidence for the presence of immunoreactive insulin in the gut, but structural data about intestinal insulins of invertebrates are not available yet.

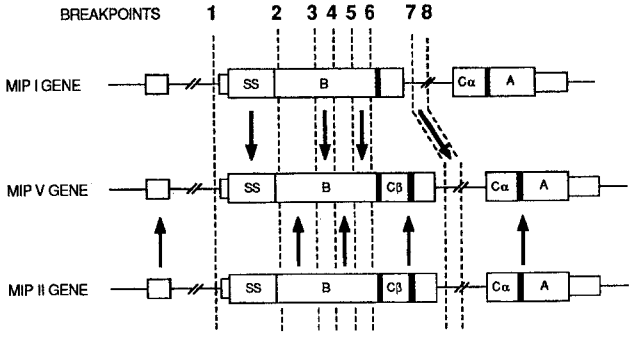

Figure 7. Schematic representation of the hybrid MIP V gene. The MIP V gene may have evolved by an exchange of stretches of the parental MIP I and II genes, very likely by intergenic reshuffling. Indicated are breakpoints, as indicated by the nucleotide sequence data, SS, signal sequence; A, A chain; B, B chain; Cα, Cα peptide; Cβ, Cβ peptide. (The MIP II gene, and therefore the MIP V gene, encode a precursor with an additional C-peptide, Cβ.)

Generation of diversity among the MIP genes: A novel mechanism for the generation of diversity among members of a neuropeptide family

An intriguing aspect of the evolution of the insulin superfamily concerns the striking difference in the degree of conservation of its members. The vertebrate insulins (and the very similar sponge insulin-related peptide) and to a lesser extent also the bombyxins are conserved, suggesting a low acceptance of mutational change. By contrast, the MIPs, like the relaxins, are widely divergent among themselves, indicating a high degree of acceptance of mutational change. Could different mechanisms account for these uneven rates of evolution in the insulin superfamily? Blundell and Wood [3] have previously pointed out that in insulins most amino acid replacements are deleterious, due to a critical interdependence of the various residues in the molecule and their strict relationship to the three-dimensional structure and the role of this structure in the physiology of (pro)insulin, e.g., synthesis, cleavage, packaging, transport, and receptor interaction. All these aspects could act as restraints during the evolution of insulin, resulting in a highly selective fixation of random amino acid replacements. This model, however, does not hold for the extensive molecular differences of MIPs and relaxins.

The MIP genes possess structural patterns that are reminiscent of important macroscale events that have taken place during evolution. The A and B chain domains of the MIP genes code for highly different peptides, while other parts, for example those encoding C peptides, are rigorously conserved. Also, in the introns, conserved and diverged regions alternate. These phenomena can be explained by macroscale events, such as the reshuffling of (relatively small) stretches (modules) of the MIP genes that have been put to the test, and found to be useful, during long periods of evolution. A convincing example is the MIP V gene, which is clearly organized as a complex mosaic pattern of nucleotide sequences derived from the MIP I and II genes. Here, reshuffling events have created MIP diversity, which is especially clear in the B chain domain of the MIP V gene (fig. 7). The duplication

of an ancestor MIP gene is very likely to have been an initial step towards MiP diversity. This early event would have released the constraint on mutational divergence, and subsequent exchanges of large parts of the MIP genes would then have significantly enhanced the rate of successful sequence variations. Whether this molecular mechanism is at the basis of the relaxin sequence diversity remains an intriguing question. The macroscale evolutionary events described for the MIP genes may be of significance for generation of diversity within other neuropeptide families as well.

Schistosomin, a peptide with a dual function: Stimulation of growth and inhibition of reproduction

Schistosomin was discovered in experiments designed to uncover the molecular basis of the endocrine interactions between the schistosome parasite *Trichobilharzia ocellata* and *L. stagnalis*. Schistosome parasites cause the widespread tropical disease schistosomiasis (bilharziasis), with freshwater snails as the intermediate hosts. In infected snails, there is an inhibition of reproductive activities and a simultaneous stimulation of somatic growth. These effects are due to schistosomin, which is specifically present in the hemolymph of parasitized animals. Further experiments have shown that schistosomin is present in the median lip nerves of normal, non-infected animals [16].

Schistosomin from the CNS of uninfected animals has been purified and sequenced. It is a 79-amino acid peptide with a complex folded structure, resulting from the presence of 8 cysteine residues, which may form four intramolecular disulphide bridges (fig. 8) [16]. In infected snails, schistosomin is released under the influence of an as yet unidentified factor produced by the developing parasite. Schistosomin, upon release, has distinct effects on growth and reproductive activities. So that the effects on reproduction can be better understood, an outline of the neuroendocrine control of reproduction is presented in the next two paragraphs. We will then discuss the interference of schistosomin with these control mechanisms.

Lymnaea, like most gastropod snails, is a simultaneous hermaphrodite. Both male and female cells, together with follicle cells and Sertoli cells, are produced in each one of the numerous acini of the ovotestis. At ovulation many oocytes are transported via the hermaphroditic duct to the female reproductive tract. After fertilization the egg cells are surrounded one by one by pervitellin fluid of the albumen gland. The fluid is rich in protein and galactogen, and serves as the source of nutrients during embryonic development. The eggs are packaged into an egg mass (or string), and during oviposition eggs are fixed to a substrate. *Lymnaea* displays an intricate pattern of behaviors accompanying egg laying or mating. Copulation is reciprocal. Autosperm is transported through the male duct and provided with secretions of the male glands. Foreign sperm received during copulations is transported through the female duct to the fertilization pocket, at the carrefour, where the male and female tracts depart from the hermaphroditic duct.

Separate male and female (neuro)endocrine control systems regulate hermaphroditism in *Lymnaea* (reviewed by Geraerts et al. [14]). A masculinizing factor (stimulating spermatogonial mitosis, as well as growth and differentiation of the male duct part of the reproductive tract) is probably present, but has not yet been structurally identified. Female cells arise by autodifferentiation, but their further maturation is controlled by the dorsal body hormone from the dorsal bodies [10], non-nervous endocrine organs that are located upon the cerebral ganglia (fig. 1). The hormone, in addition, controls the development and functioning of the female tract. The chemical nature of the hormone is still unknown. Much more is known about the peptidergic (neuroendocrine) control of egg mass formation and associated behavior. These are initiated and correlated by the caudodorsal cells, which are located in the cerebral ganglia (fig. 1). In these cells a small gene family encoding two related prohormones, the caudodorsal cell hormone (CDCH) I and II prohormones, was identified using both a CNS-specific cDNA library and a genomic library [14, 30]. From each prohormone a set of about 10 peptides can be derived. The function of some of these peptides is known. For exam-

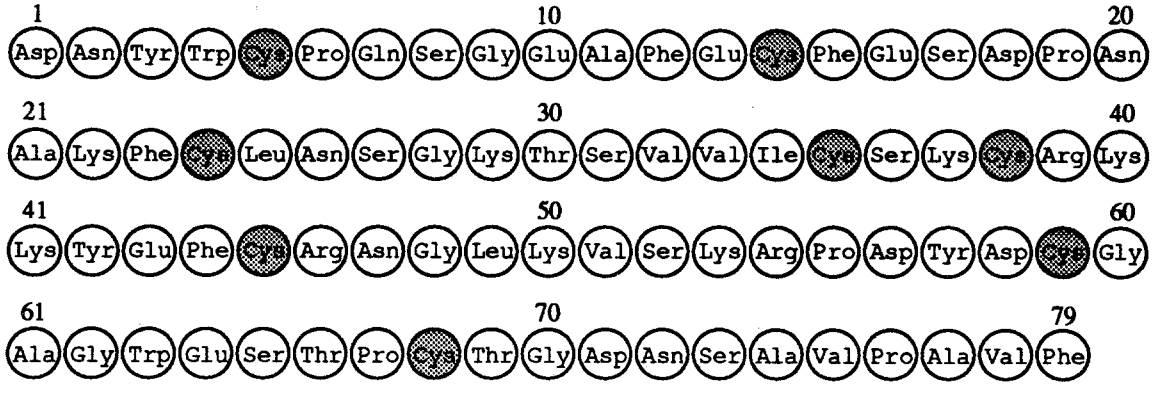

Figure 8. Primary structure of schistosomin isolated from the CNS of *L. stagnalis*. Cysteine residues are boxed.

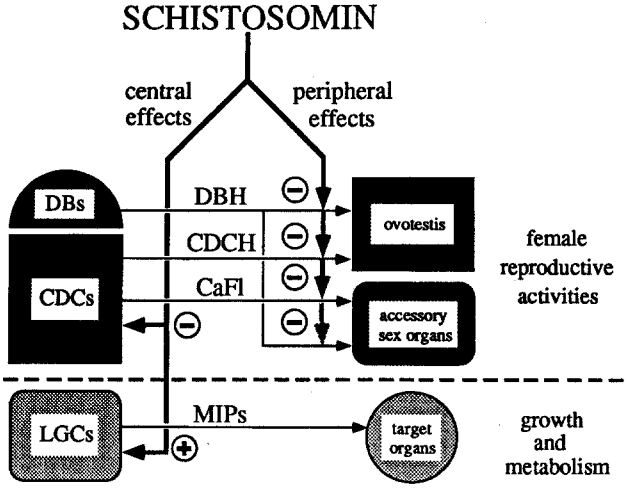

Figure 9. Schematic representation of the central and peripheral effects of schistosomin in *Lymnaea*. The effects of purified schistosomin on female reproductive activities can be distinguished as central and peripheral effects. Centrally, schistosomin reduces the excitability of the caudodorsal cells (CDCs). In the periphery, schistosomin inhibits the activities of the dorsal body hormone (DBH) and the caudodorsal cell hormone (CDCH) on the ovotestis and of calfluxin (CaFl) on the albumen gland. Furthermore, schistosomin increases the excitability of the light green cells (LGCs), which leads to release of MIPs.

ple, CDCH induces ovulation and stimulates aspects of the egg-laying behavior. Calfluxin stimulates Ca^{++} fluxes in the albumen gland, and α-caudodorsal cell peptide together with CDCH acts as an autotransmitter. Upon release, this transmitter stimulates discharge activities in the caudodorsal cells.

Schistosomin inhibits the effects of injected CDCH and calfluxin. Furthermore, it has been shown that schistosomin is capable of inhibiting the biological effects of the dorsal body hormone. These inhibiting effects on reproduction are peripheral effects and consist, probably, of interactions with CDCH, calfluxin and dorsal body hormone on the receptor level. In addition to these peripheral effects, schistosomin has clear central effects. It decreases the excitability of the caudodorsal cells, which results in the inhibition of release of the peptides producing these cells. At the same time, it increases the excitability of the LGCs, which leads to an increased release of MIPs and, thus, to an increased growth rate. The various effects of schistosomin are summarized in figure 9. The opposing effects of schistosomin on neuronal systems that control reproduction and growth suggest that the peptide may play a major role in the coordination of the two processes. And, although this has not yet been shown directly, we assume that in normal, intact snails schistosomin has a physiological role in attuning the two processes.

Concluding remarks

From the studies on the LGC peptidergic model systems several interesting conclusions can be drawn. As we have seen, a gene family coding for different though related (sets of) bioactive peptides is expressed in these neurons. This has several interesting consequences. First, the diversity and complexity of physiological processes that can be controlled by a gene family coding for different though related sets of neuropeptides is greatly expanded. Thus, such neuropeptide gene families are well suited for the regulation of complex, vital and often long-lasting life processes. Second, the MIP gene family is expressed in a stimulus-dependent way in the LGCs. A neuron's ability to express alternative peptides indicates that it has a greater number of codes for communication with its targets available to it. Therefore, this type of differential expression and release of different neuropeptides endows the neuron with a considerably increased adaptive function for information-handling. Third, in *Lymnaea* there is 'cross-talk' between the peptidergic systems controlling growth and reproduction. A clear example of the 'cross-talk' is schistosomin, which has a dual function: it excites the LGCs and inhibits the gonadotropic centers. We suggest that peptidergic neurons in the CNS of vertebrates that express multiple peptide genes in a stimulus-dependent fashion, such as peptidergic neurons in the nucleus paraventricularis, may have a similar function.

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Research Articles

Antarctic fishes survive exposure to carbon monoxide

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Abstract. The extensive in vivo conversion of haemoglobin to the carbon monoxide derivative has no discernible effect on the survival of the red-blooded Antarctic fish *Pagothenia bernacchii*. Analysis of caudally sampled blood of cannulated specimens revealed that reconversion of carbon-monoxy haemoglobin to oxyhaemoglobin was complete within 48 hours. Thus, under stress-free conditions, haemoglobin is not necessary for survival of *P. bernacchii*. Red-blooded Antarctic fishes can carry oxygen necessary for routine delivery dissolved in plasma, in a similar way to the haemoglobinless Channichthyidae, although they lack the morphological and physiological adaptations which allow the latter to prosper without any haemoglobin.
Key words. Antarctic fish; oxygen transport; haemoglobin; carbon monoxide.