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# Structure and localization of mRNA encoding a pigment dispersing hormone (PDH) in the eyestalk of the crayfish *Orconectes limosus*

Dominique P.V. de Kleijn[a], Birgit Linck[b], Jörg M. Klein[b], Wolfgang M. Weidemann[b], Rainer Keller[b] and François van Herp[a]

[a]*Department of Experimental Zoology, Catholic University of Nijmegen, Toernooiveld, 6525 ED Nijmegen, The Netherlands* and [b]*Institut für Zoophysiologie, University of Bonn, Endenicher Allee 11-13, D-5300 Bonn 1, Germany*

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The pigment-dispersing hormone (PDH) is produced in the eyestalks of Crustacea where it induces light-adapting movements of pigment in the compound eye and regulates the pigment dispersion in the chromatophores. To study this hormone at the mRNA level, we cloned and sequenced cDNA encoding PDH in the crayfish *Orconectes limosus*. The structure of the PDH preprohormone consists of a signal peptide, a PDH precursor-related peptide (PPRP) and the highly conserved PDH peptide at the carboxy-terminal end. In situ hybridization in combination with immunocytochemistry revealed four cell clusters expressing PDH in the optic ganglia of the eyestalk. Three clusters stained both with the PDH cRNA probe and the PDH antiserum, however, the perikarya in the lamina ganglionaris (LG) only stained with the PDH antiserum, suggesting the presence of a PDH-like peptide in the LG.

Pigment dispersing hormone; cDNA sequence; In situ hybridization; *Orconectes limosus* eyestalk

## 1. INTRODUCTION

The neurohormones from the X-organ sinus gland complex in the eyestalk of crustaceans are responsible for a diversity of physiological effects. Among them are chromatophore-regulating neuropeptides, like the red pigment concentrating hormone (RPCH) and the pigment dispersing hormone (PDH). The octapeptide RPCH was the first invertebrate neuropeptide of which the peptide structure was fully elucidated [1]. PDH, originally described as distal retinal pigment hormone (DRPH), is an octadecapeptide inducing light-adapting movements of pigments in the crustacean compound eye but it also regulates dispersion of pigment in chromatophores [2,3].

Immunocytochemical investigations of the optic ganglia from the crab *Carcinus maenas* and the crayfish *Orconectes limosus* suggest that PDH plays not only a role in neuroendocrine regulation but PDH-positive axons and fibers also interact with other neurons, indicating that PDH can function as a neurotransmitter or neuromodulator [4–6]. The view that PDH may not only be a chromatophorotropic neurohormone was strengthened by the isolation of a PDH homologue from heads of insects [7], in which the fast responding pigmentory effectors typical for crustaceans are lacking.

The aim of this study was to obtain additional specific tools for the investigation of PDH expression in crayfish. Therefore, we characterized the mRNA encoding PDH in *Orconectes limosus* and used the PDH cRNA probe for in situ hybridization in combination with immunocytochemistry.

*Correspondence address:* F. van Herp, Department of Experimental Zoology, Catholic University of Nijmegen, Toernooiveld, 6525 ED Nijmegen, The Netherlands. Fax: (31) (80) 652714.

## 2. MATERIALS AND METHODS

### 2.1. *Isolation and characterization of PDH-encoding cDNA*

Poly(A$^+$) RNA was isolated with guanidineisothiocyanate and oligo(dT) cellulose from medulla terminalis (MT) and medulla interna (MI) tissue of the eyestalk of *Orconectes limosus*. About 1 $\mu$g poly(A$^+$) RNA was used for constructing a cDNA library in the vector $\lambda$ZAP-II (Stratagene). About 10,000 clones of this library were screened with an *Orconectes limosus* PDH cDNA probe. This probe resulted from a PCR reaction on the MT/MI cDNA library fractions using 100 pmol of primer 1 (5′-GGGAATTCNCCNGCNTCRTTCAT-3′) based on the PDH amino acid sequence of *Uca pugilator* and 50 pmol primer 2 (5′-AGCGGATAACAATTTCACACAGGA-3′) corresponding to nucleotides 824–847 of the Bluescript II SK− plasmid. Amplification between primer 1 and 2 was performed for 5 cycles with an annealling temperature of 68°C (2 min), followed by 5 cycles at 64°C (2 min) and 40 cycles at 58°C (2 min). The denaturation step in each cycle was 40 s at 93°C, the extension step was 3 min at 72°C. The PCR product of about 400 bp was digested with *Pst*I (Boehringer Mannheim) and the 300 bp cDNA fragment and the 100 bp vector fragment were separated on a 1.5% agarose gel. The 300 bp product was then isolated from the gel by electro-elution and labeled with $^{32}$P by random priming according to standard procedures [8]. Hybridization of the replica nitrocellulose filters was performed at 42°C in 6 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodiumcitrate, pH 7.0), 1 × Denhardts solution, 25 mM sodiumphosphate buffer pH 7.0, 10% dextransulphate, 200 $\mu$g yeast tRNA/ml and 50% formamide. After hybridization the filters were washed in 2 × SSC, 0.1% sodium dodecyl sulphate (SDS) at 20°C for 10 min and subsequently washed in 2 × SSC, 0.1% SDS, 1 × SSC, 0.1% SDS and 0.25 × SSC, 0.1% SDS at 68°C for 20 min each. Hybridization-positive phage plaques were purified, and the recombinant pBluescript SK-phagemids were rescued from the bacteriophage (λZAP) clones by in vivo excision, according to the instructions of the manufacturer. Sequencing on both strands was performed with single- and double-stranded DNA using T7 DNA polymerase and the dideoxy chain termination method [9].

### 2.2. *In situ hybridization and immunocytochemistry*

Fixation of tissue and in situ hybridization has been previously described [10]. RNA probes were synthesized as run-off transcripts from linearized DNA of the *Orconectes limosus* PDH cDNA clone O1E.1 using T3/T7 RNA polymerase (Pharmacia). After 2 h at 37°C the digoxigenine labelled RNA probe was ethanol-precipitated. The RNA pellet was dissolved in 100 μl DEPC treated water. Hybridization was performed at 50°C for 16 h. After hybridization were rinsed in three changes 2 × SSC at 20°C, followed by washes at 37°C with 1 × SSC (3 × 20 min), 0.5 × SSC (2 × 20 min) and 0.2 × SSC (2 × 20 min). Immunocytochemistry, using a PDH antiserum raised in rabbits by the use of synthetic *Uca/Cancer* type PDH as antigen, was performed as described by Mangerich et al. [5].

## 3. RESULTS

### 3.1. *Isolation and characterization of MT/MI cDNA encoding* Orconectes limosus *PDH*

Screening of 10,000 clones of the MT/MI cDNA library with the 300-bp PCR fragment, corresponding to the 5′ region of *Orconectes limosus* PDH mRNA, resulted in the isolation of two hybridization-positive clones. Fig. 1 shows the nucleotide sequence of the clone with the longest insert (O1E.1) which contains a 228-bp coding region and a 131-bp 3′-untranslated region. The open reading frame codes for a protein of 76 amino acids including a 20-amino acid signal peptide [11]. The C-terminal part of the precursor protein contains an 18-amino acid peptide which is flanked on both sides by potential proteolytic processing sites (pairs of basic amino acid residues) and C-terminally by a putative amidation site. From the high degree of identity (72%–100%) between the C-terminal peptide and the primary structures of several crustacean and insect PDHs, we conclude that cDNA clone O1E.1 encodes an *Orconectes limosus* PDH preprohormone. A computer homology search revealed that the 33-amino acid long peptide preceding PDH showed no significant homology with any other protein or DNA sequence, accept with a recently cloned *Carcinus maenas* PDH preprohormone encoding mRNA [12]. The primary amino acid sequence of both preprohormones are compared in Fig. 2. Their homology is high (65% identity). Crab PDH differs only one amino acid (D for E) which gives

```
                                                          5'----CTCGAGCAAG   10
                          Signal peptide
-20                                        -10
Met Arg Ser Ala Met Val Val Leu Val Leu Val Ala Met Val Ala Val Phe
ATG CGC AGC GCC ATG GTT GTG TTG GTG CTG GTG GCA ATG GTC GCC GTC TTC          61

         -1   1                                    10
Thr Arg Ala|Gln Glu Leu Lys Tyr Pro Glu Arg Glu Val Val Ala Glu Leu
ACC CGG GCC CAG GAG CTC AAG TAT CCT GAG CGT GAG GTG GTA GCC GAA CTG          112

                                       PPRP
                      20                                  30
Ala Ala Gln Ile Tyr Gly Trp Pro Gly Ser Leu Gly Thr Met Ala Gly Gly
GCA GCC CAG ATC TAC GGG TGG CCA GGG TCC CTG GGC ACT ATG GCC GGA GGA          163

                                          PDH
                                40
Pro His|Lys Arg|Asn Ser Glu Leu Ile Asn Ser Ile Leu Gly Leu Pro Lys
CCA CAC|AAG CGC|AAC TCT GAG CTC ATC AAC TCC ATC CTG GGG CTC CCG AAG          214

    50                   •
Val Met Asn Glu Ala|Gly|Arg Arg|***
GTG ATG AAC GAG GCC GGC|AGG AGA|TAA GCGACCTCCGCATCCCTCTGCTAACTTAAGG          272

ACGAGACCAGCAGGTGTGTGACCGGAGCCTCTCACCAGCTCACACTAAGGGCGGACTGCCACCTCCT          339

ATCATGACTCTCACAGCACCAAGCAACTCACCCACTGCTGTCCCTCACCTCTGTAACAGTGTGTCTT          406

ATAGTAAAAACCTATACGAATAG(A)n----3'                                            429
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Fig. 1. Nucleotide sequence and deduced amino acid sequence of MT/MI cDNA clone (O1E.1) encoding *Orconectes limosus* PDH preprohormone. Amino acid numbering starts at the N-terminal residue of the PDH prophormone with the presumptive signal peptide sequence being indicated by negative numbering. The dot above Gly-54 indicates a potential amidation site. The two arrows indicate the dibasic processing site between the PDH (36–53) and the PDH PPRP (1–33) and the potential dibasic processing site at amino acids 55 and 56 are boxed. The signal for polyadenylation [14] is underlined.

**Signal Peptide**

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Orconectes limosus  MRSAMVV..LVLVAMVAVFTRAQELKYPERE
Carcinus maenas     MRTAVIVTMLVVVALAALLTQGQDLKYQERE
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**PPRP**

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Orconectes limosus  VVAELAAQIYGWPGSLGTMAGGPHKRNSELI
Carcinus maenas     MVAELAHEIYRVAQAPWAGAVGPHKRNSELI
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**PDH**

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Orconectes limosus  NSILGLPKVMNEAGRR
Carcinus maenas     NSILGLPKVMNDAGRR
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Fig. 2. Comparison between *Orconectes limosus* PDH preprohormone and *Carcinus maenas* PDH preprohormone [12]. Sets of identical amino acid residues are boxed. The dot indicates the difference between crayfish and crab PDH.

an identity of 94%. The preceding PDH precursor-related peptide (PPRP) of the crayfish shows an identity of 56% with the crab. The highest homology between the PPRP's is found in the N-terminal and C-terminal regions.

### 3.2. *Localization of PDH mRNA in the eyestalks*

To study the localization of PDH mRNA in the eyestalk of *Orconectes limosus*, in situ hybridization was performed using a digoxigenine labeled anti-sense cRNA probe derived from clone O1E.1 as a probe. The PDH mRNA-producing perikarya are clustered in three groups containing small and large cells (Table I). Strong signals were found in perikarya at the transition of the MT and the MI (Fig. 3A). No staining was found after hybridization with a control sense PDH probe (not shown). Immunocytochemistry using a crab PDH antiserum stained the same perikarya and some axon fibers (Fig. 3D). These labeled cell perikarya are grouped in two clusters, one dorso-laterally at the external side of the eyestalk (DLE, Fig. 3A and D), the other dorso-laterally at the internal side of the eystalk (DLI, Fig. 3B and E). A third group, expressing PDH mRNA, was found in the medulla terminalis ganglionic X-organ (MTGX, Fig. 3B and E). This cluster of 1–3 cells (Table I) showed also an immunopositive reaction (Fig. 3E). In addition small perikarya in the proximal part of the lamina ganglionaris (LG) were visualized by immunocytochemistry (Fig. 3F) while they are negative after hybridization with the PDH cRNA probe (Fig. 3C).

To examine the number and size of the perikarya, alternated sections from eyestalks of five animals where either hybridized with the PDH riboprobe or incubated with the PDH antiserum. The results are summarized in Table I. It is evident that, except for the cells in the LG, there are no striking differences between the number of perikarya stained with the riboprobe and the number of cells stained with the antiserum. The cluster at the external side of the eyestalk (DLE) contains the largests number of perikarya, while the group at the internal side (DLI) and in the MTGX is only limited to a few cells.

Table I

Number of perikarya showing a positive reaction for the pigment dispersing hormone anti-sense riboprobe or antiserum in crayfish eyestalks

| Type of perikarya cluster | Cell diameter (μm) | Average number stained with riboprobe | Average number stained with antiserum |
|---|---|---|---|
| DLE | 20–40 | 15 ± 1 | 17 ± 1 |
| DLI | 20–40 | 2 ± 1 | 2 ± 1 |
| MTGX | 30–50 | 2 ± 1 | 2 ± 1 |
| LG | 10–15 | 0 | >40 |

Average number of PDH stained perikarya in one eyestalk of *Orconectes limosus* divided into four groups according to their position in the eyestalks ($n = 5$). DLE is a cluster of PDH positive cells dorsolaterally at the external side of the eyestalk. DLI is a cluster of PDH positive cells dorso-laterally at the internal side of the eyestalk. MTGX, medulla terminalis ganglionic X-organ. LG, lamina ganglionaris.

## 4. DISCUSSION

The deduced amino acid sequence of PDH is identical to the PDH peptide of a closely related crayfish, *Procambarus clarkii* and differs only one amino acid at position 17 from *Uca pugilator* and *Cancer magister* (for review see [11]). In the preprohormone structure, the highly-conserved PDH peptide is preceded by PPRP which shows no homology to other known peptides, but appears to be a conserved peptide in Crustacea based

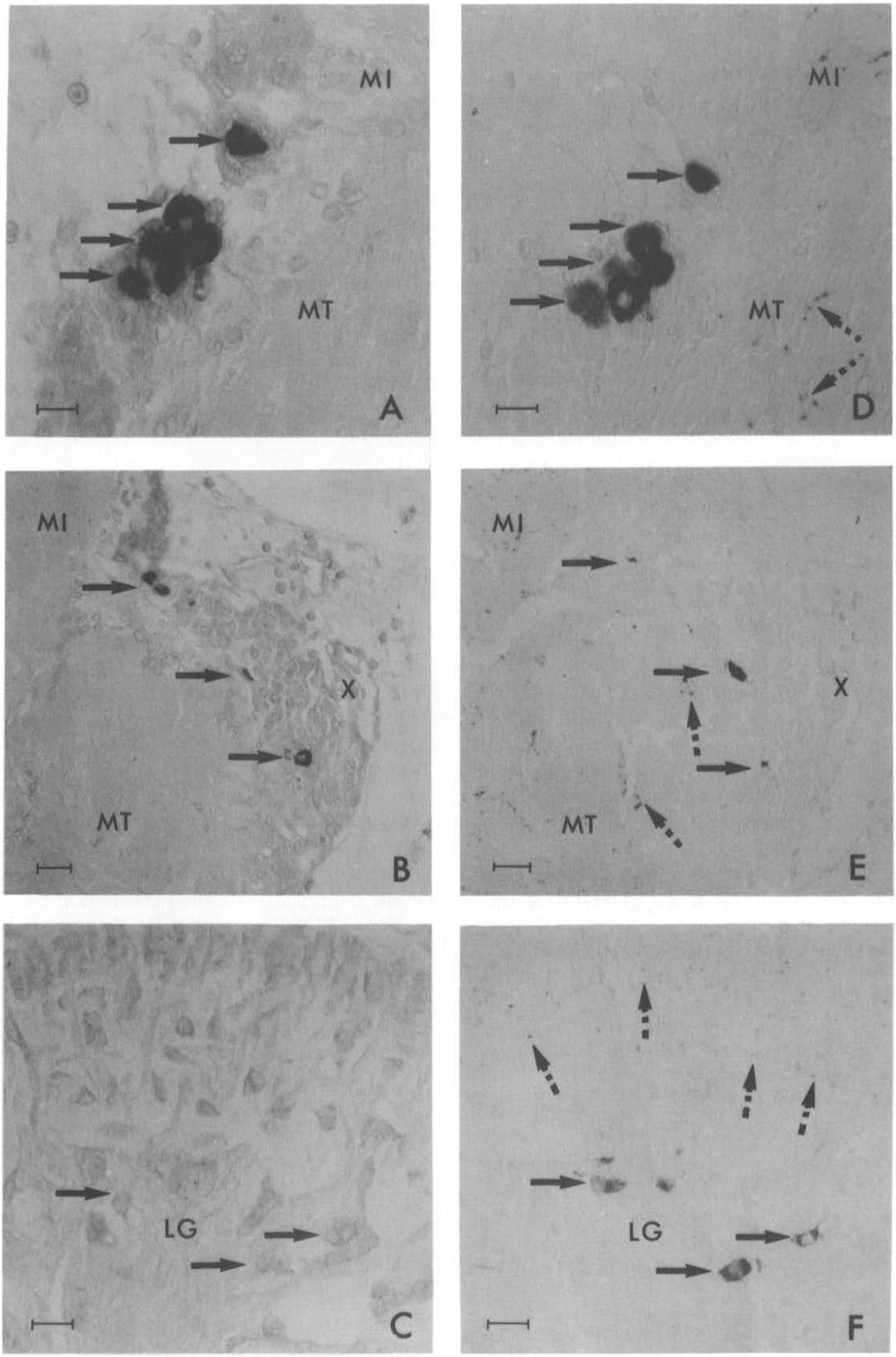
MI
MT
A
MI
MT
D
MI
X
MT
B
MI
X
MT
E
LG
C
LG
F

Fig. 3. Localization of PDH-producing cells in the eyestalk of *Orconectes limosus* by in situ hybridization and immunocytochemistry. A–C show in situ hybridization results using a digoxigenin labelled PDH encoding anti-sense cRNA probe. Positive cells are found at the transition of the MT and the MI, dorsolaterally at the external side of the eyestalk (A) and dorso-laterally at the internal side of the eystalk as well as in the MTGX (X, B). In the proximal part of the lamina ganglionaris (LG, in C) no positive reaction was found. D–F illustrate the immunocytochemical results using a crab PDH antiserum. D. Adjacent section to section in A showing double staining of perikarya containing PDH peptidergic material (D) and perikarya synthesizing PDH mRNA (A). The same situation is found for the positive cells in the serial sections of B and E at the internal side of the eyestalk and in the MTGX. In contrast, the section in F, clearly demonstrates immunopositive perikarya which show no hybridization with the PDH cRNA probe in the adjacent section in C. Scale bars are 25 μm for A,C,D and F and 50 μm for B and E. ➞ indicate stained perikarya, --➤ indicate immunoreactive fibers. In situ hybridization and immunocytochemistry are performed on 5 μm sections.

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on recent results obtained with *Carcinus maenas* [12]. The structural conservation at the N and C terminus would indicate an important function for PPRP but cDNA data for PDH preprohormones from other crustacean are necessary to confirm this observation.

The immunocytochemical data found in this study are in agreement with earlier results [5]. In addition, the immunopositive cells situated at the transition of MI and MT and in the X-organ are all positive with our cRNA probe encoding PDH. In contrast, the perikarya in the proximal part of the LG were only immunoreactive and no positive reaction was found after in situ hybridization. A possible explanation for these observations is that the cell somata contain high amounts of peptide material but no detectable PDH mRNA, indicating a difference in the rate of synthesis and storage during cell activity. Comparable results have been described for CHH- and GIH-producing cells in the X-organ of lobster eyestalks [10]. The immunopositive staining in the LG is however not stronger than in the other groups of PDH-producing cells, which clearly synthesize PDH mRNA. We thus can not exclude that a cross reactivity of the PDH antiserum with a PDH-like peptide is responsible for the LG immunostaining. The corresponding PDH-like mRNA was not found, probably because of the stringent conditions of our in situ hybridization experiments. The notion that a PDH-like peptide may occur in *Orconectes limosus* is supported by the observation that a shrimp species may contain more than one PDH [13]. In conclusion, our cloning of a PDH cDNA provides a specific tool to study the number of PDH mRNA-producing cells in crustaceans adapted to different physiological conditions, e.g. light/dark and background adaptation.

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