Signalling through pigment dispersing hormone-like peptides in invertebrates

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ABSTRACT

During recent decades, several research teams engaged in unraveling the molecular structure and the physiological significance of pigment dispersing hormone-like peptides, particularly with respect to colour change and biological rhythms. In this review, we first summarise the entire history of pigment dispersing hormone-like peptide research, thus providing a steppingstone for those who are curious about this growing area of interest. Next, we try to bring order in the plethora of experimental data on the molecular structure of the various peptides and receptors and also discuss immunolocalisation, time-related expression and suggested functions in crustaceans, insects and nematodes. In addition, a brief comparison with the vertebrate system is made.

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Abbreviations: AKH, adipokinetic hormone; aMe, accessory medulla; AVP, arginine vasopressin; BPN, brain photoreceptor neuropil; BPR, brain photoreceptor; COG, circumoesophageal ganglion; CT, circadian time; DD, continuous darkness; DH31, diuretic hormone 31; DN, dorsal neurons; DRPH, distal retinal pigment hormone; EM, external medulla; L, lamina ganglionaris; LAH, light-adapting hormone; LD, light–dark cycles; LL, continuous light; I-LNvs, large ventrolateral neurons; LNd, dorsolateral neurons; LNV, ventrolateral neurons; Lo, lobula; PACAP, pituitary adenylate cyclase-activating peptide; PDF, pigment dispersing factor; PDFAb, abdominal PDF neurons; PDFCa, PDF calyx neurons; PDF-ir, PDF immunoreactive/immunoreactivity; PDFLa, PDF lamina cells; PDFMe, PDF medulla cells; PDFR, PDF receptor; PDH, pigment dispersing hormone; PDH-ir, PDH immunoreactive/immunoreactivity; PTTH, prothoracicotropic hormone; RPCH, red pigment concentrating hormone; SCN, suprachiasmatic nuclei; SG, sinus gland; s-LNvs, small ventrolateral neurons; STNS, stomatogastric nervous system; TeTxLC, tetanus-toxin light chain; TM, terminal medulla; VIP, vasoactive intestinal peptide; VNC, ventral nerve cord; XO, X-organ.

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1. Introduction

Pigment dispersing hormone (PDH) and pigment dispersing factor (PDF) neuropeptides are conserved in a broad range of crustaceans, insects and nematodes.

In both *Drosophila melanogaster* and *Caenorhabditis elegans*, these peptidic ligands couple to class B (secretin type) G protein-coupled receptors (Mertens et al., 2005; Hyun et al., 2005; Lear et al., 2005b; Janssen et al., 2008). Both PDF and PDH are thought to be present throughout an invertebrate's life, as they are detected from the embryonic stages on, into adulthood. For crustaceans as well as for insects, the PDH/PDF-system is invariably associated with the visual system. Hence, it is not surprising that the expression levels are usually related to the changing light conditions in the environment.

In crustaceans, PDH is involved in colour changes related to the pigment dispersion of integumental pigments and shielding pigments in the compound eye. PDH also seems to be involved in regulating the sensitivity of the electrical response to photic stimulation. PDF, on the other hand, serves as an important network coordinator and output factor in the insect circadian clock. It may even regulate the plasticity of this system, as has been reported for social insects (Bloch et al., 2003). In insects, this neuropeptide is widely studied and additional functions in activity, reproduction, arousal and geotaxis have been proposed. The PDF-signal is regulated in various ways, thereby taking advantage of molecular as well as electrical signals (Nitabach et al., 2006; Miśkiewicz et al., 2008; Wu et al., 2008a). In nematodes, this neuropeptide is mainly expressed in neurons that play a role in the sensing and integration of environmental stimuli. The exact function of PDF in this phylum remains elusive; although it was shown to influence locomotor behaviour (Janssen et al., 2008, 2009). While the invertebrate PDF receptor has a clear orthologue in vertebrates, i.e. the VPAC₂ receptor, there is no clear conservation regarding the respective ligand sequences. It is nevertheless generally assumed that the functional homologue of PDF in vertebrates is vasoactive intestinal peptide, which also plays a role in the biological clock (Aton and Herzog, 2005; Vosko et al., 2007).

Here, we discuss the discovery and diversity, localisation, time-related expression, functions and receptors of the different pigment dispersing hormone-like peptides.

2. Pigment dispersing hormone in crustaceans

2.1. The discovery and diversity of pigment dispersing hormones in crustaceans

Already in 1925, the first evidence for hormones that control rapid colour changes were provided by Koller (1925, 1927). Hemolymph from a dark coloured shrimp (*Crangon crangon*) was injected into a pale one and darkening, caused by pigment dispersion, was observed soon after injection. The eyestalks turned out to be the source of this substance (Perkins, 1928). A few decades later, the complete amino acid sequence of the first pigment dispersing and light-adapting hormone was reported from the eyestalks of *P. borealis*. It was named light-adapting distal retinal pigment hormone (DRPH) (Kleinholz, 1975; Fernlund, 1976). Because DRPH also controls the epidermal pigment migration in cells of many other crustacean species, this octadecapeptide is now referred to as pigment dispersing hormone (PDH) (Rao and Riehm, 1993). Peptides with similar sequences (Table 1) are nowadays referred to as α -PDH as to distinguish them from a second peptide with pigment dispersing abilities identified from the eyestalks of the fiddler crab *Uca pugilator* (Rao et al., 1985). The sequence of this *U. pugilator* peptide differs from α -PDH at six positions and was designated β -PDH (Table 1). Additional isoforms of α - and β -PDH were identified afterwards and can all be grouped as α - or β -PDH subfamily members based on their amino acid sequence, acidity and charge similarity. The most important differences in amino acid sequence are the Gly and Met in α -PDH at position three and four whereas β -PDH contains Glu followed by Leu causing it to be more potent than α -PDH. Until to date, α -PDH has only been found in species of the genus *Pandalus* and *Macrobrachium* while β -PDH could be identified in a wide variety of crustaceans (Rao et al., 1987; Rao and Riehm, 1993; Park and Hall, 1998; Rao, 2001).

Like most neuropeptides, mature PDH originates from a larger precursor molecule. The PDH precursor contains a signal peptide, followed by a poorly conserved PDH precursor related peptide of unknown function and at the end of the open reading frame, the mature PDH flanked by either mono- or dibasic processing sites (Klein et al., 1992, 1994; De Kleijn et al., 1993; De Kleijn and Van Herp, 1995; Desmoucelles-Carette et al., 1996, 1998; Rao, 2001;

Table 1

Sequence alignment of crustacean PDH peptides. Identical amino acids are highlighted in black, conserved amino acids in dark gray. X=I or L.

Species	Peptide sequence	Reference
Crustacean α-PDH		
<i>Macrobrachium rosenbergii</i>	NSGMINSILGIPKVM A EAmide	(Rao, 2001)
<i>Pandalus jordani</i> -III	NSGMINSILGIPKVM A DAamide	(Rao and Riehm, 1989)
<i>Pandalus borealis</i>	NSGMINSILGIPRVMT E Aamide	(Fernlund, 1976)
<i>Pandalus jordani</i> -II	NSGMINSILGIPRVMT E Aamide	(Rao and Riehm, 1989)
Crustacean β-PDH		
<i>Cancer borealis</i> -II	NSELINSILGL S RLMNEAamide	(Ma et al., 2009b)
<i>Cancer productus</i> -II	NSELINSILGL S RLMNEAamide	(Fu et al., 2005b; Hsu et al., 2008; Hsu et al., 2010)
<i>Callinectes sapidus</i> -II*	NSELINSILGL S ALMNEAamide	(Klein et al., 1994)
<i>Pacifastacus leniusculus</i>	NSELINSILGLPKVMNDAamide	(Rao and Riehm, 1993)
<i>Uca pugnator</i>	NSELINSILGLPKVMNDAamide	(Rao et al., 1985)
<i>Neohelice granulata</i>	NSELINSILGLPKVMNDAamide	n.d.
<i>Carcinus maenas</i>	NSELINSILGLPKVMNDAamide	(Klein et al., 1992; Lohr et al., 1993; Ma et al., 2009a)
<i>Cancer productus</i> -I	NSELINSILGLPKVMNDAamide	(Fu et al., 2005b; Hsu et al., 2008)
<i>Cancer magister</i>	NSELINSILGLPKVMNDAamide	(Kleinholz et al., 1986)
<i>Cancer borealis</i> -I	NSELINSILGLPKVMNDAamide	(Ma et al., 2009b)
<i>Callinectes sapidus</i> -I	NSELINSILGLPKVMNDAamide	(Klein et al., 1994; Mohrherr et al., 1990)
<i>Astacus leptodactylus</i>	NSE XXXXXX PKVMNDAamide	own unpublished results
<i>Orconectes immunis</i>	NSELINSILGLPKVMNEAamide	(Rao and Riehm, 1993)
<i>Orconectes limosus</i>	NSELINSILGLPKVMNEAamide	(Bulau et al., 2004; DeKleijn et al., 1993)
<i>Procambarus clarkii</i>	NSELINSILGLPKVMNEAamide	(McCallum et al., 1991)
<i>Litopenaeus vannamei</i> -B	NSELINSILGLPKV E IDAamide	(Desmoucelles-Carette et al., 1996; Ohira et al., 2006)
<i>Penaeus japonicus</i> -I	NSELINSILGL S PKVMTDAamide	(Ohira et al., 2002; Yang et al., 1999)
<i>Pandalus jordani</i> -I	NSELINSILGLPKVMTDAamide	(Rao and Riehm, 1989)
<i>Penaeus japonicus</i> -II	NSELINSILGLPKV E IDAamide	(Ohira et al., 2002; Yang et al., 1999)
<i>Litopenaeus vannamei</i> -A	NSELINSILGL S PKVMNDAamide	(Desmoucelles-Carette et al., 1996; Ohira et al., 2006)
<i>Penaeus aztecus</i>	NSELINSILGLPKVMNDAamide	(Phillips et al., 1988 fide Rao and Riehm, 1989)
<i>Armadillidium vulgare</i>	NSELINSILGL S PRV N NAamide	(Rao and Riehm, 1993)
<i>Daphnia pulex</i> *	NSELINSILGLPR F PKVamide	(Gard et al., 2009)
<i>Orconectes limosus</i> -B	NSELINAILG S PT F FGVamide	(Bulau et al., 2004)
<i>Orconectes limosus</i> -C	NSELINAILG S PT F MGVamide	(Bulau et al., 2004)

*Amino acid sequence derived from cDNA precursor sequence (ClustalW2 and BOXSHADE 3.21).

Chuman et al., 2002; Ohira et al., 2002; Yamano and Unuma, 2006; Hsu et al., 2008; Gard et al., 2009). The amino acid sequence of various PDH peptides in crustaceans turned out to be largely identical which results in biological cross-reactivity. Bioassays like the injection of fractionized eyestalk extracts (Rao et al., 1985, 1987) to induce pigment dispersion in eyestalkless crabs provided a good start for the discovery of chromatophorotropin-like peptides. Applications like multi-dimensional HPLC separations, more advanced mass spectral techniques and the construction and screening of EST databases contributed to an even more extended list of PDH peptides in crustaceans. Crustaceans including *Callinectes sapidus*, *Cancer productus*, *Litopenaeus vannamei* (also known as *Penaeus vannamei*), *Penaeus japonicus* (also known as *Marsupenaeus japonicus*), *Orconectes limosus* and *Pandalus jordani* turned out to express multiple forms of β -PDH or even both α - and β -PDH as in the case of *P. jordani* (Rao and Riehm, 1989, 1993; Mohrherr et al., 1990; De Kleijn et al., 1993; Klein et al., 1994; Desmoucelles-Carette et al., 1996; Yang et al., 1999; Rao, 2001; Ohira et al., 2002, 2006; Bulau et al., 2004; Fu et al., 2005a,b; Hsu et al., 2008). The kuruma prawn *P. japonicus* expresses two different β -PDH peptides. They were initially purified from sinus gland (SG) extracts and bioassays showed that both peptides were able to cause pigment dispersion in all four types of chromatophores. *Pej*-PDH-II showed to be almost ten-fold more potent than *Pej*-PDH-I for erythrophores, xanthophores and melanophores. Erythrophores and xanthophores were the most sensitive of the four chromatophores (Yang et al., 1999). Fu and co-workers revealed the presence of two distinct β -PDH peptides in *C. productus* (Fu et al., 2005b). Molecular cloning of the cDNAs revealed one transcript (*prepro- β -pdh I*) encoding the authentic β -PDH (NSELINSILGLPKVMNDAamide; Rao et al., 1985), from then on referred to as β -PDH I. Two other transcripts were both found to encode a second peptide, NSELINSLGLSLRMNEAamide, from then on called β -PDH II (Hsu et al., 2008, 2010). These cDNAs

encoded distinct PDH-precursor-related peptides and were therefore named *prepro- β -pdh Ila* and *prepro- β -pdh Ilb*. The presence of multiple PDH peptides raises the question whether or not this is a way to create a redundant PDH system or whether the peptides have different functions. Double *in situ* hybridisation and direct tissue mass spectrometry suggested that the two isoforms have distinct functions. While only *Canpr*- β -PDH II was detected in the neurosecretory SG, indicating that it acts as a neurohormone, *Canpr*- β -PDH I was found in all other parts of the eyestalk suggesting that it has a function as a local neuromodulator or neurotransmitter (Hsu et al., 2008) (see also Section 2.2.1). The *C. productus* β -PDH II is almost identical to *C. sapidus* β -PDH II except for position 11 and 13 (Table 1). Despite the sequence similarity, their functions are predicted to be different. As opposed to *C. productus*, β -PDH II in *C. sapidus* is thought to act as a local neurotransmitter/modulator and β -PDH I is found in the SG indicating that it acts as a neurohormone (Klein et al., 1994). These hypothetical functions based on localisation represent the only attempts to clarify the presence of multiple PDH peptides in one species.

Two of the three *O. limosus* β -PDH peptides (PDHB and PDHC) distinguish themselves from the other known crustacean β -PDH peptides with a Val at the C-terminus instead of an Ala (Bulau et al., 2004) (Table 1). Recently, the *Daphnia pulex* PDH precursor could be identified by means of an *in silico* analysis of an EST database. *D. pulex* PDH is predicted to also have a Val as a C-terminal amino acid (Gard et al., 2009). The biological function of this distinct structure unfortunately remains elusive.

Small variations in amino acid sequence can cause significant changes in potency of the peptides depending on the position of the amino acids. By testing different forms of PDH in the *U. pugnator* pigment dispersion bioassay, it can be concluded that the two substitutions at position eight and eleven are not critical for PDH potency. On the contrary, positions three, four and seventeen

influence PDH potency significantly since α -PDH with Gly and Met at positions three and four respectively is less potent than β -PDH. Substitution of Leu at position four by Ile does not prevent receptor activation as insect PDH-like peptides like the *Romalea microptera* PDF are able to induce pigment dispersion in *U. pugnator* (Rao et al., 1987). Although it seems that especially the N-terminal part of the peptide is critical for its potency, Glu at position 17 instead of Asp causes a significant decrease in potency in the crustacean bioassay (Rao and Riehm, 1989) (Table 1).

Although a vast collection of different PDH-peptides have been synthesised and tested, and the effects and the potencies of these peptides have been studied thoroughly, there is still a lack of knowledge concerning the actual PDH receptor(s) and cellular pathway(s) involved in pigment dispersion. A single mRNA and derivative amino acid sequence of a predicted PDH receptor in *P. japonicus* is known without any additional information (GenBank AB478163.1). It has been suggested that PDH signalling comprises adenylate cyclase, cAMP and protein kinase A (Nery and Castrucci, 2002).

2.2. Pigment dispersing hormone localisation

When synthetic α - and β -PDH became available for the production of antibodies, immunocytochemical PDH localisations were conducted (Schueler et al., 1986; Dirksen et al., 1987). Since PDH was known to be an eyestalk hormone, localisation studies were initially carried out in the optic ganglia of the eyestalks. Shortly after, PDH-immunoreactive (PDH-ir) structures in other regions of the cerebral ganglion (also known as the brain or supraoesophageal ganglion) and in the ventral nerve cord (VNC) and stomatogastric nervous system (STNS) were explored (Mangerich and Keller, 1988; Mortin and Marder, 1991). It turned out that the majority of PDH-ir cells and fibers are located in the eyestalks and the median protocerebrum (as is also the case for insects as described in Section 3.2).

Sequentially, we will discuss PDH localisation in the cerebral ganglion (optic ganglia and median protocerebrum), the VNC and the STNS.

2.2.1. PDH localisation in the cerebral ganglion of crustaceans

In general, the decapod brain consists of three regions called the protocerebrum, deutocerebrum, and tritocerebrum, all with a very complex internal organisation of neuropil regions and tracts (Fig. 1A). PDH localisation is particularly observed in the protocerebrum which consists of the optic ganglia, lateral protocerebrum and median protocerebrum. Among neuropil regions in the eyestalks, are the lamina ganglionaris (L), external medulla (EM) and lobula (Lo) (Fig. 1A). Another neuropil of interest regarding PDH localisation is the terminal medulla (TM) in the lateral protocerebrum. The median protocerebrum contains the protocerebral bridge, central body, posterior medial protocerebral neuropil and the anterior medial protocerebral neuropil which is connected to the TM through the protocerebral tract (also known as the optic nerve or optic tract) (Sandeman et al., 1992; Böhm et al., 2001; Kirby and Nusbaum, 2007).

α -PDH immunoreactive structures are specifically observed in the eyestalks of *Pandalus* species. α -PDH-ir is co-localised with β -PDH-immunoreactivity (β -PDH-ir) in *Pandalus danae* eyestalks (Phillips et al., 1990). Actually, the majority of PDH-ir studies have been carried out with β -PDH antibodies and detailed descriptions of β -PDH-ir in the brains of *Carcinus maenas*, *Cancer productus*, *Cherax destructor*, *Orconectus limosus*, *Homarus americanus*, *Cancer pagurus* and *U. pugnator* are now available (Mangerich et al., 1987; Dirksen et al., 1987; Hsu et al., 2008; Harzsch et al., 2009). The most striking similarities are the putative innervation of the neurohemal SG by neurons

located between the Lo and the TM as seen in Astacurans as well in Brachyuran species (Fig. 1B and D) together with β -PDH-ir in the median protocerebrum (Fig. 1C).

β -PDH localisation studies in Astacuran species were conducted in *Procambarus clarkii*, *Cancer destructor*, *Homarus americanus* and *O. limosus* (Mangerich et al., 1987; Dirksen et al., 1987; Mangerich and Keller, 1988; De Kleijn et al., 1993; Sullivan et al., 2009; Harzsch et al., 2009). Harzsch and colleagues nicely illustrate β -PDH-ir in eyestalks, median protocerebrum and VNC (see Section 2.2.2) in the lobster *H. americanus* (Harzsch et al., 2009). The β -PDH-ir pattern in the eyestalks of *H. americanus* consists of intense labelling of somata between the EM and TM, fiber networks in the EM and TM and fibers terminating in the SG (Fig. 1B). A clear bundle of fibers runs from the somata (C, C' and C'') between the EM and TM towards the TM and leaves the eyestalk towards the median protocerebrum via the protocerebral tract. The same somata also seem to have axons that enter the sinus gland (SG) and additionally extend their axons to form fiber networks in the EM. Some other somata (A and B) associated with the TM also send their axons to the median protocerebrum through the protocerebral tract. Some of the axons running from the eyestalks to the median protocerebrum continue their path around the oesophagus and towards the VNC (not shown). Additionally there are two pairs of β -PDH-ir somata located in the anterior part of the median protocerebrum and two other large labelled somata between the protocerebral plexus which shows β -PDH-ir and the posterior medial protocerebral neuropil (without PDH-ir) (Harzsch et al., 2009).

P. clarkii as well as *C. destructor*, shows intense characteristic β -PDH-ir in the anterior regions of the median protocerebrum. In both species, there are also large, paired β -PDH-ir somata in Cluster 6 which extend posteriorly through the brain and into the circumoesophageal connectives. In *C. destructor*, the most intense β -PDH-ir is found in the brain photoreceptor neuropils (BPNs) (Fig. 1C). The BPNs are innervated by axons extending from the extraretinal brain photoreceptor (BPR) which is not β -PDH-ir. Interestingly, as described in more detail in Sections 2.3 and 2.4.4, expression of β -PDH in these regions of the brains of both *P. clarkii* and *C. destructor* varies during the day (Sullivan et al., 2009).

β -PDH expressing cells in the eyestalks of *O. limosus* are also located at the transition of the Lo and TM. The X-organ (XO) contained only two or three cells, which are probably of minor significance. In addition, small perikarya in the proximal part of the L were found by immunocytochemistry. This might be a result of cross-reaction, since these cells were not found by cRNA probes (De Kleijn et al., 1993). Again, several somata with β -PDH-ir were located in the anterior part of the median protocerebrum, sending axons towards the optic neuropil and into the eyestalk (Mangerich and Keller, 1988).

PDH localisation in the nervous system of Brachyurans is most extensively studied in the crab *C. productus* and moreover, β -PDH-ir was verified by *in situ* hybridisation with isoform specific riboprobes and mass spectrometry. Since β -PDH-ir in *C. productus* is similar to that in other Brachyurans, we will only discuss β -PDH localisation in the Brachyuran *C. productus* (Hsu et al., 2008).

The eyestalk ganglia of *C. productus* contain several β -PDH-ir somata in the L, between the Lo and TM and in the XO (Fig. 1D). Somata between the Lo and TM give rise to axons that innervate neuropils in the EM, Lo and TM and some also arborize within the SG. It is not clear what parts are innervated by axons departing from the β -PDH-ir somata in the XO but it is clear that it is not the SG. In the median protocerebrum, intense β -PDH-ir could be detected in two somata of Cluster 6. These project to and ramify in the optic neuropil in the median protocerebrum and travel through the optic nerve to the TM. One fiber travels from the optic neuropil to both circumoesophageal connectives of the STNS (see Section 2.2.3) (Hsu et al., 2008).

Fig. 1. Representation of β -PDH immunoreactivity in the cerebral ganglion of several crustacean representatives. (A) Schematic overview of the decapod brain. The decapod cerebral ganglion consists of the proto- (PC), deutero- (DC) and tritocerebrum (TC). The protocerebrum can be divided in the eyestalks with neuropil regions called the lamina ganglionaris (L), external medulla (EM) and lobula (Lo), the terminal medulla (TM) in the lateral protocerebrum and the median protocerebrum (MPC). (B) Representation of β -PDH immunoreactivity in the eyestalks of the Astacuran *H. americanus* at 60% of embryonic development. Axons extending from a pair of β -PDH-ir neurons (A) and a single β -PDH-ir cell (B) associated with the terminal medulla (TM), target the median protocerebrum through the protocerebral tract (PT). Another group of β -PDH-ir cells (C, C' and C'') have axons extending to the external medulla (EM), TM, medulla satellite neuropil (S) and PT where they exit the eyestalk towards the brain. Additionally, axons from group C somata are thought to target the sinus gland (SG) as well. The lamina ganglionaris (L) is also innervated by β -PDH-ir fibers. Retina (R). Adapted with kind permission from Springer Science+Business Media (Harzsch et al., 2009, Fig. 1c). (C) Representation of β -PDH immunoreactivity in the median protocerebrum of the Astacuran *C. destructor*. A summary of β -PDH-ir in the median protocerebrum of *C. destructor*, based on detailed micrographs and descriptions published by Sullivan et al. (2009). The typical β -PDH-ir pattern consists of two large labelled cell bodies in Cluster 6 (6). A fiber extends from these cells and travels posteriorly into the circumoesophageal connectives (not shown). The arrowhead indicates one of the paired brain photoreceptor neuropils (BPNs) which are clearly labelled. Large axons travelling through the protocerebral tract (PT) are also labelled. The olfactory lobe (OL) and accessory lobe (AL) are not labelled but their position is included for orientation. (D) Representation of β -PDH immunoreactivity in the eyestalk of the Brachyuran *C. productus*. A summary of β -PDH-ir in the eyestalk of *C. productus*, based on detailed micrographs and descriptions published by Hsu et al. (2008). β -PDH-ir is observed in cell bodies in the lamina ganglionaris (L), between the lobula (Lo) and the terminal medulla (TM) and in the X-organ (XO). Additionally, the sinus gland (SG) is labelled. Arrowheads indicate axon bundles (1) innervating the SG and leaving via the optic nerve, (2) Lo and EM and (3) innervating the SG and not travelling through the optic nerve.

Like several other crustaceans, *C. productus* expresses more than one PDH isoform (Table 1) (Fu et al., 2005b; Hsu et al., 2008). In order to distinguish between the two isoforms of *C. productus* and confirm the results of the antibody staining, double *in situ* hybridisation with riboprobes was carried out to localise *prepro- β -pdh I* and *II* mRNA. *Prepro- β -pdh I* mRNA was indeed found in the cell bodies of the L, a cluster of cell bodies between the Lo and TM and in the XO. *Prepro- β -pdh II* mRNA was found in other Lo/TM cell bodies and a single XO cell body where it is co-localised with *prepro- β -pdh I*, but not in the L (Hsu et al., 2008).

Finally, Matrix-assisted laser desorption/ionisation Fourier transform mass spectrometry (MALDI-FTMS) was used to analyze several eyestalk tissues of *C. productus*. This resulted in a positive identification of β -PDH I in the L, EM, Lo, TM and XO. β -PDH II on the other hand, was specifically localised in the SG suggesting that *C. productus* β -PDH II can act as a neurohormone. *C. productus* β -PDH I is however thought to act as a local neurotransmitter and/or modulator (Hsu et al., 2008).

So far, PDH expression patterns have mostly been studied in the adult nervous system of crustaceans. Surprisingly, most of the PDH-positive structures in adult animals can already be found in early embryological stages as was shown in the Astacuran *H. americanus* (Harzsch et al., 2009). Unfertilised *Carcinus maenas* oocytes showed that PDH is expressed from maternally derived PDH mRNA during embryonic development. Immunocytochemical experiments showed that the peptide appears as soon as one to two days after the onset of eye formation (when the embryo is already developed for about 50%). The functional significance of

the early PDH presence remains elusive but the authors suggest it may point to circadian clock-driven processes in the embryo (Chung and Webster, 2004; Harzsch et al., 2009).

Some species exhibit either PDH expression in the eyestalks or the median protocerebrum. For example in the pandalid *P. japonicus*, RT-PCR demonstrated that PDH expression was only present in the eyestalk and not in the brain, thoracic ganglia, abdominal ganglia, hindgut and hepatopancreas (Ohira et al., 2002). PDH-like staining within the CNS of the copepod crustacean *Calanus finmarchicus* with an antibody against *U. pugilator* β -PDH (Dirksen et al., 1987) is limited to one pair of somata in the protocerebrum and the axonal projections emanating from them (Sousa et al., 2008).

2.2.2. PDH localisation in the ventral nervous system

PDH-ir in the ventral nervous system is generally less pronounced compared to the eyestalks and median protocerebrum.

The ventral nervous systems of the Astacurans *O. limosus* and *H. americanus* show similar PDH-ir (Mangerich and Keller, 1988; Harzsch et al., 2009). Fibers extending from the protocerebrum reach the circumoesophageal ganglia (COGs) through the circumoesophageal connectives. PDH-ir positive perikarya are found in the suboesophageal ganglion of *O. limosus* but PDH could not be located in the thoracic or abdominal ganglia. Axons showing PDH-ir travel towards the caudal end of the VNC with extensive branching in all thoracic and abdominal ganglia. Similarly in *H. americanus*, large medial PDH-ir somata in the median protocer-

ebrum of *H. americanus* send axons towards the VNC via the circumoesophageal connectives together with neurites extending from the eyestalks (Section 2.2.2). Branching of these axons results in a plexus with PDH-ir in the COGs. More posteriorly, PDH-ir is observed throughout the entire VNC with extensive branching in the ganglia of the VNC (Harzsch et al., 2009).

In the Brachyuran *C. maenas*, PDH-ir in the VNC is different as the ventral ganglia are differently organised (Mangerich and Keller, 1988). *C. maenas* contains more somata with PDH-ir in its condensed thoracic ganglion with their axons forming a plexus in the posterior part of the thoracic ganglion (Mangerich and Keller, 1988).

2.2.3. PDH localisation in the stomatogastric nervous system

The STNS of crustaceans consists primarily of the interconnected stomatogastric ganglion, oesophageal ganglion and the paired commissural ganglia (COGs) and motor neurons innervating the muscles of the stomach (Fig. 2). It generates rhythmic motor patterns responsible for moving, chewing and filtering food particles (Christie et al., 2010).

Mortin and Marder characterised β -PDH distribution in the Palinuran *Panulirus interruptus*, the Astacurans *H. americanus* and *P. clarkii* and the Brachyurans *C. borealis* and *C. antennarius* by using an antibody raised against β -PDH from *U. pugnator* (Dircksen et al., 1987; Mortin and Marder, 1991). In all five species, β -PDH-ir somata, neuropils and fibers were observed in the COGs though in different positions within the COG (Fig. 2). Additional PDH-ir localisation varied among the different species and sometimes also between the more closely related species as is the case with *C. borealis* and *C. antennarius*. *C. antennarius* is the only species who exhibited PDH-ir structures in the oesophageal ganglion and inferior oesophageal nerve. In *C. antennarius*, six neurons located in the COGs were PDH-ir. Of these, two were cross-connecting the two COGs, indicating a possible function as bilateral communicators. Two homologous PDH-ir cells were identified in *C. borealis* but the crossing PDH-ir fibers were absent. Two other Brachyuran species, *C. productus* and *C. sapidus*, show similar distribution of PDH-ir in the STNS but without the cross-connecting fibers in the OG and ion (Hsu et al., 2008).

P. interruptus showed a high concentration of PDH-ir fibers in the neuropil region of the stomatogastric ganglion, originating from the COG. This intensive staining of neuropil synapses suggests a local modulatory function of PDH in the stomatogastric ganglion. *H. americanus* and *P. clarkii* showed extremely faint staining in the neuropil region of the stomatogastric ganglion. Several other neuropeptides proved to modulate the rhythmic motor patterns generated by the stomatogastric ganglion but it still remains unclear if PDH is biologically active in the STNS (Christie et al., 2010; Mortin and Marder, 1991).

2.3. Time-related expression

PDH in crustaceans is released upon illumination. Additionally, experiments with *Procambarus bouvieri* showed that a circadian

rhythm of PDH concentration in the nervous system persists under constant dark conditions (Aréchiga and Mena, 1975). Recently, the intensity of PDH-ir in the brains of *C. destructor* and *P. clarkii* was shown to be in phase with their locomotor activity rhythms (Sullivan et al., 2009). Both crayfish are more active during the night compared to the day, but show distinct peaks of activity. Without anticipation, *C. destructor* rapidly increases its activity level with the onset of darkness, followed by a gradual decrease of activity towards the onset of day. *P. clarkii* shows two distinct peaks of activity: one at the onset of darkness and one before the beginning of the day. PDH expression in Cluster 6 cell bodies in *C. destructor* follows the activity pattern by increasing almost two-fold after the onset of darkness, followed by a gradual decrease over the next 7–8 h. PDH expression in the BPNs of *P. clarkii* also matches the specific activity pattern with a significant increase preceding both night and day (see also Section 2.4.4, circadian entrainment) (Sullivan et al., 2009).

2.4. Function and regulation

2.4.1. Integumental colour change

Colour change is a phenomenon observed in many crustaceans and has been studied for more than a century. Colour changes can be brought about by the multiplication of chromatophores. These are specialised pigment-bearing cells with branched cytoplasmic processes. In crustaceans, there are four types of chromatophores: erythrophores, xanthophores, leukophores and melanophores, containing respectively red, yellow, white and black or brown granules (Fingerman, 1970; Josefsson, 1983). Most of them are part of the integument and are located in the connective tissue below the epidermis. Slow colour changes can be observed in areas with a transparent or thin exoskeleton. Division of the chromatophores causes the total amount of pigment to increase and results in a darker colour. However, rapidly reversible integumental colour changes are brought about solely by intracellular concentration or dispersion of pigment granules. Among crabs, colouration may vary in function of social behaviour such as mating or as a result of e.g. background colour, temperature, stress or moulting (e.g. Little, 1967; Takeda, 2006; Silbiger and Munguia, 2008; Detto et al., 2008). The best studied colour change is the endogenous mechanism that especially fiddler crabs (*Uca*) use, to darken during the day and lighten at night. The darker colour acts as a natural sunscreen, providing protection against intense sunlight during the day. For example, melanophores and erythrophores of *U. pugnator*, *U. rapax* and *U. maracoani* and melanophores of *U. pugnax*, *U. mordax* and to a much lesser extent *U. thayeri*, continue to alternate dispersing and concentrating their pigments under constant darkness (Brown, 1950; Hines, 1954; Barnwell, 1963; Thurman, 1988). Apart from these *Uca* species, the blue crab *C. sapidus* and the crab *Neohelice granulata* (formerly known as *Chasmagnathus granulatus*) also show persistent rhythms of pigment migration in melanophores (Fingerman, 1955; Granato et al., 2004).

Fig. 2. Overview of β -PDH positive structures in the stomatogastric nervous system of several decapods crustaceans. Circumoesophageal ganglion (COG), stomatogastric ganglion (STG), oesophageal ganglion (OG), superior oesophageal nerve (son), stomatogastric nerve (stn), intersection of oesophageal-, superior oesophageal- and stomatogastric nerve (on/son/stn), circumoesophageal connective (cc), inferior oesophageal nerve (ion).

The origin of this endogenous rhythm can be found in the cyclic synthesis and release of RPCH and PDH. Granato and co-workers showed that the melanophores of *N. granulata* additionally respond to light itself. This primary response amplifies the black pigment dispersion in response to PDH and can be observed even when the PDH trigger is lost. Additionally, the responsiveness of the melanophores varies through the day, with a larger response to PDH during the day compared to the night (Granato et al., 2004).

2.4.2. Dispersion of shielding pigments in the eyes

Pigment movements also take place within the ommatidia of the compound eyes of crustaceans. These eyes contain an elaborate system of pigments for screening and reflection of light and are able to reversibly adapt to bright and dim light (Fig. 3) (reviewed by Meyer-Rochow, 2001). Decapod ommatidia typically contain a lens-shaped cornea that is part of the exoskeleton. Immediately below the cornea are two corneagenous cells which secrete the cornea. The crystalline cone below the corneagenous cells consists of four crystalline cells. Most decapods have superposition (dark-adapted or nocturnal) eyes which are able to adjust their sensitivity, albeit to a limited extent, to bright light. In superposition eyes, the crystalline cone is connected to the light-sensitive rhabdome located at the base of the ommatidium by a cone stalk which transmits the light (Fig. 3). This rhabdome is actually a gathering of rhodopsin containing microvilli which arise from seven circular reticular cells. Light passing the cone stalk is able to reach neighbouring rhabdomes of other ommatidia which causes a blurred image but achieves a maximum detection of dim light. To be able to adapt to changing light intensities, the reticular cells contain granules with retinal shielding pigments called the proximal pigments. Two cells surrounding the crystalline cone and tracts of the ommatidia contain the so-called distal pigments. Additionally, Astacuran decapod crustaceans such as shrimps, crayfish, prawns and lobsters have reflecting pigments at the base of the ommatidia. Both the proximal and distal screening pigment granules can either be dispersed in a light-adapted position where they prevent the light rays from reaching neighbouring photo-sensitive rhabdomes, or they can be aggregated in a dark-adapted position leaving the rhabdomes exposed to light (Fig. 3). In fact, these changes follow a circadian pattern (Bennitt, 1932b; Hernández-Falcón et al., 1987).

The migration of the proximal and distal pigment granules is controlled by distinct mechanisms. On one hand, the proximal pigment granules are able to quickly disperse without the presence of the SG as was shown in *Cambarus* and *Procambarus* (Kleinholz, 1949; Olivo and Larsen, 1978). Some authors have suggested though, that the cells containing the proximal pigments are also hormonally controlled (Bennitt, 1932a,b; Welsh, 1939; Fingerman et al., 1962). On the other hand, the circadian movement of the distal and reflecting pigment was shown to be under control of an eye-stalk hormone in *Palaemonetes* (Welsh, 1930; Kleinholz, 1934, 1936). This hormone turned out to be the same hormone that causes rapid integumental colour changes, i.e. PDH, also named light-adapting hormone (LAH) or distal retinal pigment hormone (DRPH) (Fernelund, 1976).

2.4.3. Retinal response

Not only does PDH influence the movement of the shielding pigments, it also seems to have several effects on the photo-transduction process itself. Crustacean photoreceptor cells depolarise upon illumination. This can be recorded by means of an electrode penetrating the cell. In *P. clarkii*, exogenous applied PDH causes the amplitude of the depolarisation to diminish in an irreversible way. However, the amplitude of this effect depends on the circadian time suggesting a higher affinity or maybe more

Fig. 3. Generalized representation of an Astacuran ommatidium. On the left: proximal and distal pigments in position for light adaptation (LA). On the right: proximal and distal pigments in position for dark adaptation (DA). Cornea (co), corneagenous cell (chc), crystalline cone (cc), distal pigment (dp), crystalline tract (ct), proximal pigment (pp), rhabdome (rh), reflecting pigment (rp).

receptors present at certain moments. Also, the duration of depolarisation shortens at circadian time 6 (CT 6) but becomes longer at CT 18 (Verde et al., 2007). This phase changing ability of PDH suggests that PDH acts as a circadian output factor just like PDF does in insects (Helfrich-Förster et al., 2000).

The electrical response to photic stimulation of the compound eye can also be recorded by means of an electroretinogram for which an electrode is placed on the surface of the eye. The magnitude of this response displays a clear circadian rhythm which has been extensively studied, particularly in crayfish (Aréchiga et al., 1993; Aréchiga and Rodríguez-Sosa, 1998). Exogenous PDH also showed to act as a non-photoc synchroniser of this rhythm inducing phase advances or delays, depending on the time of application (Verde et al., 2007).

2.4.4. Circadian entrainment

Like all organisms on earth, crustacean species are exposed to daily changes of the environment caused by the daily rotation of our planet. Most animals and plants have a circadian (circa = around, dies = day) clock that enables them to anticipate these changes by adapting physiological processes and/or behaviour. Apart from rhythmic colour change and pigment dispersion (as described in the previous paragraphs), crustaceans also display circadian locomotor activity (a complete overview of crustacean circadian rhythms can be found in Table 1 of Strauss and Dircksen,

2010). Although various studies have been carried out to analyse activity rhythms in crustaceans, it remains unclear how this circadian clock exactly functions. A central pacemaker has not yet unequivocally been identified and the question remains whether the crustacean clock exists of a single or of multiple dispersed pacemakers. Recently, Sullivan and co-workers did succeed in linking PDH to circadian entrainment in two Astacurans: *P. clarkii* and *C. destructor* (Sullivan et al., 2009).

In addition to the compound eyes, crayfish have extraretinal photoreceptive systems called brain photoreceptors (BPRs) and caudal photoreceptors (located in the sixth abdominal ganglion in the VNC) (Prosser, 1934; Wilkens and Larimer, 1972; Sandeman et al., 1990). These BPRs of *C. destructor* are excellent circadian photoreception cell candidates since they express cryptochrome, a photoprotein connecting directly to the circadian clock of plants, mice and insects and also histamine which is a neurotransmitter in several arthropod photoreceptor systems (Somers et al., 1998; Thresher et al., 1998; Stuart and Callaway, 1991; Stanewsky et al., 1998; Emery et al., 1998; Stuart et al., 2007). In order to examine the function of the BPRs in circadian entrainment, the compound eyes and caudal photoreceptors of *P. clarkii* and *C. destructor* were removed and the effects on photoentrainment of locomotor activity rhythms were investigated (Sullivan et al., 2009). Apparently, BPRs suffice to entrain locomotor rhythms to photic stimuli. Interestingly, the BPRs project to the BPN which is extensively innervated by PDH-ir neurons. As mentioned in Section 2.3, PDH levels in both *C. destructor* and *P. clarkii* vary simultaneously with the locomotor activity rhythms of each of these crayfish. The PDH-ir neurons in Cluster 6 of *C. destructor* might provide a direct link between light stimuli captured by the BPR and behavioural output (locomotion) as these interneurons connect to motor centers for locomotion in the suboesophageal and thoracic ganglia (Sullivan et al., 2009).

3. Pigment dispersing factor in insects

3.1. The discovery and diversity of pigment dispersing factor peptides in insects

Following the characterisation of PDH peptides in crustaceans, peptide extracts from insects showed to be able to induce pigment dispersion in the chromatophores of the crab *U. pugilator* and were named pigment dispersing factors (PDFs) (Dores and Herman, 1981; Mohrher and Rao, 1985; Rao et al., 1987). The first non-

crustacean pigment dispersing substance to be characterised, was the lubber grasshopper *R. microptera* PDF. The *R. microptera* peptide shows 78% homology with β -PDH from *U. pugilator* (Rao et al., 1987). Insect PDF peptides are closely related to β -PDH as they consist of 18 amino acids, a N-terminal Asn, a C-terminal Ala (*Nasionia vitripennis* excluded) and at least 50% sequence similarity (Tables 1 and 2). Similarly to crustacean PDH, insect PDF originates from a larger precursor molecule consisting of a signal peptide, a PDF-associated peptide and the mature PDF flanked by mono and/or dibasic cleavage sites.

Some beetles, e.g. *Tribolium castaneum*, seem to lack PDF (Li et al., 2008). A tBLASTn with the *D. melanogaster* PDF receptor and *C. elegans* PDF receptors as query sequences does however reveal a possible PDF receptor in *T. castaneum* (XP_971738.1).

3.2. Pigment dispersing factor localisation

As a result of growing interest in the PDF peptide, a vast variety of insects adds up to the list of localisation studies. Originally, these experiments were performed using antibodies raised against β -PDH; this approach is still valuable as a first indication of PDF localisation in species with yet unknown PDF sequences. Such results should nevertheless be handled with caution, as they may slightly differ from true PDF patterns due to cross-reaction.

While some common conclusions can often be made considering the localisation of PDF in the central nervous system (Table 3), species specific details can differ substantially. In the following description, we would like to point out some recurring themes, without ignoring the diversity observed over the different species. Both brain and abdominal PDF neurons have been identified, supporting a true neurohormonal role for this peptide.

3.2.1. PDF neurons in the insect brain

3.2.1.1. Three distinct clusters in hemimetabolan representatives. The hemimetabolan species so far studied all seem to have three distinct PDH or PDF immunoreactive (PDH-ir or PDF-ir) clusters in the optic lobe as hallmark. Multiple representatives of Phasmida, Dictyoptera, Dermaptera, Othoptera and Hemipteroid origin have been probed for putative PDF neurons in numerous experiments, but many await neuroanatomical identification in order to validate a hypothesis for most members of the hemimetabolan lineage.

Table 2

Sequence alignment of insect PDF peptides. Identical amino acids are highlighted in black, conserved amino acids in dark gray. X=I or L.

Species	Peptide sequence	Reference
Insect PDF		
<i>Drosophila virilis</i> *	NSELINSLLSLPKNNMNDAAmide	n.d.
<i>Phormia regina</i> *	NSELINSLLSLPKNNMNDAAmide	(Matsushima et al., 2004)
<i>Drosophila melanogaster</i>	NSELINSLLSLPKNNMNDAAmide	(Park and Hall, 1998)
<i>Apis mellifera</i>	NSELINSLGLPKNNMNDAAmide	(Boerjan et al., 2010)
<i>Nasonia vitripennis</i> *	NSELINSLLSLPKNNMNTAmide	n.d.
<i>Anopheles gambiae</i> *	NSELINSLLSLPKTMNDAAmide	n.d.
<i>Musca domestica</i> *	NSELINSLLSLPKSMNDAAmide	n.d.
<i>Bombyx mori</i> *	NSELINSLLSLPKDMNDAAmide	(Roller et al., 2008)
<i>Acheta domesticus</i>	NSELINSLGLPKVLNDAAmide	(Rao and Riehm, 1989)
<i>Gryllus bimaculatus</i> *	NSELINSLGLPKVLNDAAmide	(Chuman et al., 2002; Singaravel et al., 2003)
<i>Meimuna opalifera</i> *	NSELINSLGLPKVLNDAAmide	(Sato et al., 2002)
<i>Romalea microptera</i>	NSELINSLGLPKLLNDAAmide	(Rao et al., 1987)
<i>Schistocerca gregaria</i> *	NSELINSLGLPKLLNDAAmide	Personal communication J. Tobback
<i>Leucophaea maderae</i>	NSELINSLGLPKVLNDAAmide	(Hamasaka et al., 2005)
<i>Periplaneta americana</i>	NSELINSLGLPKVLNDAAmide	(Mohrher et al., 1991)
<i>Carausius morosus</i>	NSELINSLGLPKVLNDAAmide	(Mohrher et al., 1994)
<i>Blattella germanica</i> *	NSELINSLGLPKVLNDAAmide	n.d.

*Amino acid sequence derived from cDNA precursor sequence (ClustalW2 and BOXSHADE 3.21).

Table 3

Overview of anatomical terms and orthologous structures. (A) Different terminology for insect structures. (B) Orthologous PDH/PDF-positive cells in arthropods.

(A) Terminology		
Distal posteriodorsal = (dorsal) PDFLa		
Distal posterioventral = (ventral) PDFLa		
Proximal frontoventral = PDFMe		
(B) Orthologous structures in arthropods		
<i>L. maderae</i> (Hemimetabola)	<i>D. melanogaster</i> (Holometabola)	<i>H. americanus</i> (Crustacea)
Anterior PDFMe		
–4 large	4 l-LNvs	Group C neurons
–4 medium	4 s-LNvs	
–4 small	–	
Posterior PDFMe	–	
PDFLa (ventral & dorsal)	–	Cells in proximal part of lamina ganglionaris

The very first localisation experiment in insects was performed in the grasshopper *R. microptera*, where PDH-ir was detected in three cell groups in the optic lobes and in widely distributed arborisations in the supraoesophageal ganglion (Zahnow et al., 1987). Comparative studies further confirmed the appearance of these three clusters in other species (Nässel et al., 1991; Homberg et al., 1991b; Sato et al., 2002; Sehadová et al., 2003; Honda et al., 2006; Wen and Lee, 2008). Two cell groups are localised at either the posterior dorsal or posterior ventral edge of the lamina and are therefore together known as the PDFLa cells or separately as the distal posteriodorsal and posterioventral clusters. The third group can be observed at the anterior edge of the medulla and is referred to as the PDFMe cells or the proximal frontoventral cluster (Fig. 4 and Table 3) (Nässel et al., 1991; Homberg et al., 1991b; Sehadová et al., 2003).

Although the number of observed PDH-ir PDFLa cells varies between species, they are of equal dimensions and invariably project into the lamina, accessory medulla (aMe) and distal medulla. The network of fibers that spreads over the medulla ends in the first optic chiasm or in the lamina (Fig. 4) (Homberg et al., 1991b; Sehadová et al., 2003).

While generally regarded homologous group of neurons over the investigated species, the PDFMe neurons show more variation regarding the size of the somata and the individual innervation patterns in the midbrain. Noteworthy are outgrowths in the lateral protocerebrum and in two areas close to the mushroom bodies (Homberg et al., 1991b), as well as a connection between the two bilaterally located PDH-like systems (Sehadová et al., 2003) (Fig. 4).

The presence of additional PDH-ir neurons in proto-, deutero- and tritocerebrum as well as suboesophageal ganglion, frontal ganglion, recurrent nerve and corpora cardiaca differs among species. Detailed functional consequences are not known (Sehadová et al., 2003; Abdelsalam et al., 2008). In some species PDH-ir in the posterior optic tubercles suggests an indirect connection between PDFMe neurons and the central complex (Homberg et al., 1991b).

We would like to give a slightly more detailed description of the optic lobe PDH-ir architecture in the cockroach *Leucophaea maderae*, since it is by far the best studied hemimetabolan model with regard to PDF localisation. Its PDF expression pattern is often compared to the PDF-system of the holometabolan fruit fly *D. melanogaster* (Table 3). In the optic lobe of *L. maderae*, four groups of PDH-ir neurons can be identified: anterior and posterior PDFMe neurons, and ventral and dorsal PDFLa neurons. Constructing a 3D-model of the cockroach aMe and its PDH-ir innervation, three subgroups of the twelve anterior PDFMe neurons could be identified: four with large, four with medium and four with small somata. The large anterior PDFMe cells might synaptically connect to serotonergic neurons and are proposed as homologues of the *D. melanogaster* l-LNvs (Fig. 4), whereas the medium PDFMe probably interact with different

Fig. 4. Schematic overview of recurring PDF-regions in the hemimetabolan brain. Three clusters of PDF-producing cells (dPDFLa, dorsal PDFLa cells; vPDFLa, ventral PDFLa cells; PDFMe, PDFMedulla cells) provide ramifications in lamina (La), medulla (Me) and lobula (Lo; LoVT, lobula valley tract) of the optic lobe and the lateral regions of the protocerebrum (ILP, inferior lateral protocerebrum; VLP, ventral lateral protocerebrum), and connect both hemispheres (AOC, anterior optic commissure; POC, posterior optic commissure; Ca, calyx of the mushroom body; SOG, suboesophageal ganglion; triangles represent cell clusters and not individual neurons).

partners and are related to the *D. melanogaster* s-LNvs. Only the small PDFMe have projections that are limited to the ipsilateral optic lobe, and do not have *D. melanogaster* LNv counterparts. Contralateral communication through large and medium PDFMe neurons is thought to serve in a circadian coupling pathway (Reischig and Stengl, 2003b; Reischig et al., 2004).

Although there are some general aspects, not all representatives of even the same order invariably fulfil them. An example can be found in a study of PDF immunoreactivity in a number of termite species. Despite some differences, there are general aspects for most termite species: there is but a limited number of PDF-ir neurons in the PDFMe cluster, but the tritocerebrum and frontal ganglion are greatly innervated and there are PDF-ir perikarya observed in the suboesophageal ganglion (Závodská et al., 2008). Note that the presence of the PDFMe cluster only, is reminiscent of a holometabolan PDF-architecture (see Section 3.2.1.2). The PDF-cells of the suboesophageal ganglion supply, amongst other targets, fibers to the corpora cardiaca. This innervation pattern is rare in non-termite insects, indicating an order-specific characteristic of PDF-staining. It should be noted that absence of PDH-ir neurons in the optic lobe is extremely rare, yet it has been observed in two termite species, *Reticulitermes flavipes* and *Macrotermes carbonarius*, indicating species-specific deviations of the general pattern (Závodská et al., 2009). It would certainly be interesting to further investigate the consequences of this absence in termites.

Some studies report on subcellular localisation of PDF. In *G. bimaculatus*, PDF is not only detected in the cytoplasm of medulla and lamina neuropils, but can also be observed in the nucleus of at least some of the PDFLa cells. This is supported by the precursor sequence, which differs slightly from the common PDF sequences, in that it does not have a clear signal sequence, but a nuclear localisation sequence instead (Chuman et al., 2002). It is possible that in this species, PDF performs an additional role in the nucleus. Such nuclear localisation was on the other hand not observed in the last summer cicada *Meimura opalifera* (Sato et al., 2002), indicating species-specific differences.

Another molecular property that is associated with localisation is isoform formation. Although the existence of PDH isoforms was well known for crustaceans, it was not until 2006 that this suggestion was made for insects. Honda and colleagues used a variety of antibodies raised against *G. bimaculatus* PDF, each one recognizing a different epitope. Immunostaining then revealed that the 'standard' PDF is

present in the aMe, PDFMe cells, the inner PDFLa cells and their axons; while an isoform was detected in the PDFLa outer cells and a varicose arborisation in the lamina. As the outer PDFLa cells do not provide *pdf* mRNA, this peptide may be a product of another gene. It is however possible that there is no different gene, but that some antibodies do not recognize a structural change of the peptide (Honda et al., 2006). The information is nevertheless interesting, as isoforms create many new physiological possibilities that are yet to be investigated for insects.

Based on these localisation studies, hypotheses on PDF functions in insects have been made, such as control of the endocrine route (Wen and Lee, 2008; Vafopoulou et al., 2010) and functions in the circadian clock mechanism (see Section 3.4).

3.2.1.2. PDFMe neurons in holometabolan representatives. All Endopterygota studied so far display PDH/PDF-ir in the PDFMe cluster. PDFLa neurons seem to be absent in these insects. With the fruit fly as leading animal model for decades, PDF neurolocalisation data are yet available in great detail. Additional studies conducted in other holometabolan species broaden the view with some deviations from the general PDF pattern.

Three distinct clusters of PDH-ir neurons were originally identified in the brain of the fruit fly *D. melanogaster*, of which only one remains visible in adult flies (Helfrich-Förster, 1997): the *D. melanogaster* PDFMe neurons are observed throughout development into the adult stage. These neurons are also named the ventrolateral neurons, and are divided into two distinct groups, according to the size of their somata: there are five small (s-LNVs) and four large ventrolateral neurons (l-LNVs) (Fig. 5). Only four s-LNVs are PDH-ir. They all project into the superior protocerebrum and have dendrites in the larval optic lobe neuropil, and its adult counterpart, the aMe. The l-LNVs differentiate during metamorphosis, during which connections to the aMe and medulla become visible. The l-LNVs additionally connect both brain hemispheres by projecting contralaterally via the posterior optic tract (Helfrich-Förster, 1997) (Fig. 5). Some l-LNV neurons were first thought to send collaterals into the dorsal brain, but this was not observed in later studies (Park and Griffith, 2006; Helfrich-Förster et al., 2007). This means that the l-LNVs are the only clock neurons not projecting into the dorsal brain (Helfrich-Förster et al., 2007). A recent study reporting on mRNA populations enriched in the LNV neurons as compared to the entire head, supports the observation

that PDF presence in adult brains is restricted to these neurons solely (Kula-Eversole et al., 2010). Similarly, two groups of at least four lateral neurons in each optic lobe of the housefly *Musca domestica* express *pdf*. They appear to correspond to the small and large LNVs of *D. melanogaster* (Matsushima et al., 2004). It should be noted that the presence of PDF in the s-LNVs of the housefly is subject of debate (Bahn et al., 2009). Eight PDFMe cells are also present in each hemisphere of the blowfly *Protophormia terraenovae*. Four of them have large somata, the other four somata are of a smaller diameter (Nässel et al., 1991) (Fig. 5). In blowfly larvae, however, only seven PDFMe were observed by Hamanaka et al. (2009). Two large axons originating from two of the large somata represent the connection with the lamina of the optic lobes. Other projections are observed in three medulla layers, in a region adjacent to the mushroom body calyx, in the posterior optic tract and medial above the oesophageal foramen (Nässel et al., 1991) (Fig. 5). For fruit flies, it has been proposed that the l-LNV receive ipsilateral extraretinal photic input from the Hofbauer-Büchner eyelet (Helfrich-Förster et al., 2007). Similarly, direct connections of such an eyelet with PDF-ir neurons have been described for the blowfly (Yasuyama et al., 2006). The bumblebee and honeybee PDFMe neurons provide limited innervation of lamina, medulla and the proximal region of the posterior lobula. The protocerebral neuropils are delineated by PDH-ir commissural fibers (Bloch et al., 2003; Weiss et al., 2009).

For the fruit fly, an additional cluster of two to four PDH-ir neurons was initially detected close to the calyces of the mushroom bodies in adults, and therefore named PDFCa neurons. They were thought to send axons via the median bundle to the tritocerebrum (Helfrich-Förster and Homberg, 1993; Helfrich-Förster, 1997). Unlike for the LNV neurons, however, these results could not be confirmed by anti-DrpPDF staining or *in situ* hybridisation (Park and Hall, 1998; Renn et al., 1999; Park et al., 2000). This cluster was therefore considered to result from cross-reaction (Renn et al., 1999; Park et al., 2000).

The second cluster of fruit fly PDH-ir neurons in the tritocerebrum displays immunoreactivity during a short period in development: from about halfway metamorphosis until four days after eclosion. The somata are located ventrally from the oesophageal foramen and their fibers run dorsally in the median bundle to the pars intercerebralis and lateralis (Helfrich-Förster, 1997; Renn et al., 1999; Park et al., 2000). The decrease of the PDF-

Fig. 5. Schematic overview of prominent PDF regions in the brain of two holometabolan representatives. Both the fruit fly and the blowfly reveal a fan-like innervation structure of the optic lobe (Me, medulla) and provide PDF-positive neurites in the lateral region (LH, lateral horn neuropil adjacent to the calyx of the mushroom body) and between both hemispheres (POT, posterior optic tract). In *D. melanogaster*, the medullar PDF clusters (PDFMe) are known as large and small ventrolateral neurons (l-LNV and s-LNV). The blowfly additionally has PDF-producing neurons in the posterior dorsal (PD) and medial (PM) regions of the brain (MP, medial portion of the brain) and has PDF-positive neurites extending to and innervating the lamina region (La) (Lo, lobula; aMe, accessory medulla; Ca, Calyx of the mushroom body; OF, oesophageal foramen; SOG, suboesophageal ganglion; triangles represent cell clusters and not individual neurons).

signal after eclosion most likely reflects a complete retraction of the neurons themselves (Fernández et al., 2008). In general, more PDF-ir neurons are found in *D. melanogaster* larvae compared to adults and staining decreases with age in adult flies, while the pattern remains the same (Helfrich-Förster et al., 2000).

In the blowfly *P. terraenovae*, apart from PDFMe staining, an additional pair of PDH-ir cell bodies is located close to the posterior midline (Nässel et al., 1991); this pair is also observed in larval flies and has axons that cross the midline and project ventrally along the foramen of the oesophagus, terminating in the dorsal region of the suboesophageal ganglion (Hamanaka et al., 2009) (Fig. 5). Another eight cell bodies could be detected in the lateral neurosecretory cell group of the superior protocerebrum (Nässel et al., 1993) (Fig. 5). Projections are observed in the median bundle, and some even seem to run posteriorly into the cervical connective, which is the spinal cord equivalent descending from the fly's brain. Three pairs project to the corpora cardiaca and retrocerebral complex (Nässel et al., 1993). In larvae, a pair of ascending fibers, possibly derived from cells in the thoracic-abdominal ganglion, terminates close to the optic neuropil. PDF-ir fibers, likely derived from pars lateralis cells, were furthermore detected in the wall of the aorta, but not in the ring gland. PDF could be released from the aorta in the hemolymph (Hamanaka et al., 2009).

The limited number and considerable dimensions of the moth *Manduca sexta*'s neurosecretory cells allowed an early identification of PDH-ir neurons. A dorsomedial cluster of brain neurosecretory cells with prominent arborisations in the tritocerebrum was found to express a PDH-like substance. Interestingly, in this species no PDH-ir cells were observed in the aMe (Homberg et al., 1987, 1991a; Wise et al., 2002).

On the cellular level, Miśkiewicz et al. clarified the long-presumed presence of PDF in the dense core vesicles, studying both the fruit fly and the housefly, and verified that the peptide is released in a non-synaptic, paracrine way (Miśkiewicz et al., 2004). They could also show that PDF is the only peptide present in the housefly's PDH-ir varicosities of the medulla (Miśkiewicz et al., 2008). This sheds a new light on PDF localisation studies in other insects that might need to be reinvestigated at subcellular level. Colocalisation with RFamide-like peptides was observed in *M. sexta* by means of immunostaining with gastrin/cholecystokinin antibodies (Homberg et al., 1987, 1991a) and it seems that PDF colocalises with RFamide-like peptides and corazonin at least in some neurons in the brain of the blowfly (as observed with antibodies against CCK-8, corazonin and FMRFamide) (Hamanaka et al., 2007, 2009). Many studies have suggested that FMRFamide, a neuropeptide that is – like PDF – thought to regulate circadian clock output, is localised with PDF in some neurons (Homberg et al., 1991a; Nässel et al., 1993; Meinertzhagen and Pyza, 1999; Pyza and Meinertzhagen, 2003; Settembrini and Villar, 2005). More recent findings contradict the presence of FMRFamide in PDF-neurons, at least for the fruit fly and the housefly. As these experiments were conducted in great detail, it is assumable that FMRF-neurons and PDF-neurons comprise separate, though closely associated neuron sets (Matsushima et al., 2007).

Based on localisation patterns, some indications concerning structure–function relations can be derived. For example, the L1 and L2 first order interneurons are the most important target interneurons of the photoreceptor terminals in the housefly *M. domestica*. While PDH-ir cells influence L1 and L2, direct synaptic connections between both have not been described. It has therefore been proposed that lamina glial cells might be involved in transmitting the signal (Meinertzhagen and Pyza, 1996), or that the peptide diffuses to its site of action (Meinertzhagen and Pyza, 1999). In *D. melanogaster*, medulla neuropil glial cells are similarly thought to be involved in certain functions of the PDF-system and are in close contact with the terminals of the PDF-positive I-LNvs

(Górska-Andrzejak et al., 2009). Since there is also evidence for serotonin to influence the housefly L1/L2 and some serotonin-positive processes run along PDH-positive ones, these neurons could work together in the circadian regulation (Meinertzhagen and Pyza, 1996). Interestingly, blowfly serotonin immunoreactive neurons equally show the same general expression pattern as PDH-ir neurons, though comprising a distinct group of cells. This observation is also valid for the cockroach *L. maderae*, suggesting that both molecules might be functionally coupled, with common follower neurons (Nässel et al., 1991).

Other neurotransmitters influencing the housefly L1 and L2 cells are histamine, glutamate and GABA. Only the latter is proposed as another candidate for circadian regulation, a major function of PDF (Meinertzhagen and Pyza, 1999).

3.2.2. Abdominal PDF neurons in insects

PDF is a neuropeptide for which there is often information available both on local functions in the brain as well as on probable hormonal roles within the same animal. A more detailed study on the central action of PDF by electrophysiologically investigating the abdominal PDF neurons (PDFAb) of *Locusta migratoria* revealed two types of PDF-ir neurons in the abdominal ganglia. Lateral neurons project to heart nerves and perisymphatic organs, which are the release sites for neurosecretory products of the VNC. Therefore, these PDFAb neurons are ideally situated to support a hormonal role for the neuropeptide. The less complex median neurons innervate the hindgut and posterior midgut. PDF, however, does not influence hindgut contractility, but seems to be released into the hemolymph, again indicating an additional hormonal function for this neuropeptide (Persson et al., 2001). In general, considerable variation exists between the insect PDFAb neurons. For *D. melanogaster*, only neurons similar to the median neurons have been observed while closely related blowflies may express the peptide in more cells. Moths have some PDFAb neurons, but cockroaches and crickets seem to have none (Homberg et al., 1991a; Persson et al., 2001; Wise et al., 2002; Wen and Lee, 2008).

The third cluster of PDH-ir neurons of *D. melanogaster*, located in the eighth abdominal neuromere of the ventral ganglion, comprises the PDFAb neurons. They undergo severe reorganisations throughout development, but PDF-production is also observed after these neuronal changes. Axons from the PDFAb neurons innervate the most caudal part of the midgut and the hindgut. Since they are closely associated with the circular muscles of the posterior midgut, they might provide direct innervation. Abundant expression of the PDF receptor (PDFR) in the crop furthermore suggests that PDF could be released into the hemolymph (Veenstra et al., 2008).

In the thoracic-abdominal ganglion of the blowfly, all of the 36 neurons label weakly with β -PDH antiserum, except for three pairs of ventral abdominal neurons that show strong PDH-ir. These three pairs are also observed in the fruit fly. The PDFAb cells innervate all abdominal neuromeres and send projections to the hindgut (Nässel et al., 1993).

3.3. Time-related expression

Because of the tight association between PDF and the biological day-night rhythm, several researchers have looked into the possibility of daily PDF or PDF-associated expression rhythms. Obviously, there are more biological clock-related changes than those described below. Here, we will focus on PDF.

For the blowfly *P. terraenovae* and the cockroach *L. maderae*, no differences of PDH-ir could be observed over the course of the day (Nässel et al., 1991; Hamasaka et al., 2005). One should keep in mind that more subtle differences below the detection level cannot be excluded. Similarly, the hemolymph PDF levels of *L. migratoria*

showed no significant difference between morning and evening. This should be interpreted separately from the information concerning the brain, as abdominal PDF may function as a clock-independent circulating neurohormone, in addition to its local function in the CNS (Persson et al., 2001). In contrast to these constant PDF levels, both cricket nymphs and adults show daily changes in PDF content of the optic and cerebral lobes (Abdelsalam et al., 2008). Cycling PDF levels have also been observed in *Rhodnius prolixus* (Vafopoulou et al., 2010).

Both the housefly *M. domestica* and the fruit fly *D. melanogaster* have been studied into more detail concerning PDF-associated rhythms. The housefly exhibits two circadian rhythms in its visual system that are dependent on PDF input (Pyza and Meinertzhagen, 1996; Balys and Pyza, 2001). Firstly, a fluctuation in synaps number is observed between photoreceptor terminals and their target interneurons L1 and L2, and in feedback synapses formed by L2. Secondly, the axons of both interneurons seem to change in size during the day, being at their largest during the day, and shrinking by night (Meinertzhagen and Pyza, 1996, 1999). Although these changes are circadian, they are also directly influenced by light. PDF itself is in the housefly cyclically released from varicosities of PDH-ir terminals in a paracrine, non-synaptical fashion (Miśkiewicz et al., 2008). For the fruit fly, Northern blotting *pdf* mRNA revealed a slight, though never significant cycling over the course of a day and was therefore considered stable (Park and Hall, 1998). Interestingly, although the RNA is produced in a constant fashion (Park and Hall, 1998; Park et al., 2000), the peptide levels at the axon terminals oscillate with a daily pattern: they are most abundant in the early morning and reach their lowest levels about 1 h after lights off (Park et al., 2000). Cycling peptide levels were observed in the s-LNVs, but not in the l-LNVs or their processes. It is thought that this reflects a daily rhythm of peptide processing, transport or release (Park et al., 2000). Thus not only *pdf* RNA and peptide localisation, but also the temporal expression within one neuron is different (Helfrich-Förster et al., 2000). In addition to changes in peptide levels, the dorsal axon terminals of the s-LNVs undergo daily morphological changes, being more complex during the day than during the night. The rhythm persists under constant darkness (DD), indicating the true circadian nature. This clock-controlled neuron plasticity could participate in the regulation of downstream information transmission (Fernández et al., 2008). As in the housefly, daily changes in L1 and L2 size and shape are similarly observed for *D. melanogaster* (Meinertzhagen and Pyza, 1999). In contrast to the observations for the brain, PDF levels seemingly remain constant in the abdominal cells of the ventral ganglion (Blau and Young, 1999).

Whereas it is clear that PDF itself is crucial for normal circadian behaviour, there are studies that argue that PDF cycling *per se* might not be as important for daily rhythms as assumed, since adult flies without PDF cycling remained rhythmic in DD (Kula et al., 2006). Also, this could explain why constant amounts of membrane-tethered PDF can produce (though abnormal) rhythmic behaviour in a *pdf* deletion mutant (Choi et al., 2009; Taghert, 2009). So far, there is no single explanation for these phenomena.

3.4. Function of the PDF system

3.4.1. The visual system and the biological clock

It is impossible to discuss PDF function and regulation without a basic understanding of the molecular nature of the circadian clock. In an extremely simplified, but for this purpose sufficient view, we can state that most organisms endogenously generate 24 h rhythms based on negative feedback loops of gene transcription activation and inhibition. For the fruit fly and many other insects, heterodimers of the transcription factors

CLOCK (CLK) and CYCLE (CYC) activate the transcription of *period* (*per*) and *timeless* (*tim*) genes, whose protein products impair the CLK/CYC binding to the DNA, thus negatively regulating their own transcription. Apart from many posttranscriptional regulations, a second loop including the genes *pdp-1* (PAR domain protein 1) and *vrille* acts to stabilize this oscillator. Clock neurons are typically the PER-producing neurons, which do not invariably colocalise with PDH-ir neurons (Závodská et al., 2003).

Historically, the search for PDF function in insects originates in the tight association of the peptide with the visual system. Because the involvement of PDF in the insect eye pigment migration has not been reported and epithelial chromatophores are absent in these animals, a different function for the neuropeptide must be present (Chen et al., 1992; Rao and Riehm, 1993; Pyza and Meinertzhagen, 1997 and references therein).

PDF-positive neurons transfer and modulate rhythmic and photic signals, and connect both accessory medullae (aMe) (Nässel et al., 1993; Tomioka and Abdelsalam, 2004; Yasuyama et al., 2006; Helfrich-Förster et al., 2007). Whereas for most studied species PDF is a crucial but not the sole signalling molecule required for normal circadian locomotor activity (Taghert et al., 2001), this may yet be the case for the nocturnal German cockroach (Lee et al., 2009).

Much of the early work on circadian rhythms has been performed on cockroaches, where it was shown that cutting the optic stalks causes degeneration of PDH-ir terminals, accompanied by the loss of circadian locomotor rhythms. Regeneration of these terminals is furthermore associated with a reappearing circadian locomotor rhythm (Stengl and Homberg, 1994). Similarly, changes in PDFMe neuron arborisations accompany splitting in the locomotor rhythm in *D. melanogaster* mutants, meaning that the rhythm decomposes into its components, thus connecting the PDFMe neurons to the circadian clock (Helfrich-Förster and Homberg, 1993). It should be noted that the PDFMe neurons are not the clock neurons in all insects, for example not in the cricket *G. bimaculatus* (Okamoto et al., 2001).

The first direct physiological evidence for a circadian PDH function in insects was also achieved for the cockroach. After injecting synthetic PDH in the accessory medulla region of *L. maderae*, Petri and Stengl (1997) were able to elicit an amount- and phase-dependent resetting of the circadian locomotor rhythm. PDH in insects could therefore, either as a nonphotic input signal or as an integral part of the circadian pacemaker, couple the bilaterally paired circadian pacemakers. Additionally, they showed that the phase delay amplitude resulting from PDH injections was similar to that caused by serotonin (Petri and Stengl, 1997). Later, actions of PDF, FMRFamide and serotonergic neurons have been identified in the tight coupling between both brain hemispheres (reviewed by Helfrich-Förster, 2004). Reischig and Stengl were furthermore able to restore circadian locomotor activity by ectopically transplanting the adult accessory medulla into optic lobeless cockroaches. Restoring the rhythm was accompanied by formation of PDH-ir neurons from the graft to the protocerebral targets. These experiments proved that at least the large- and medium-sized PDH-ir neurons are pacemaker neurons in the accessory medulla of *L. maderae* (Reischig and Stengl, 2003a).

In addition to circadian locomotor activity regulation, a PDF-dependent mechanism of the l-LNVs is part of a light-activated arousal circuit, modulating behavioural arousal and sleep in the fly brain (Sheeba et al., 2008a). The l-LNV arousal circuit probably uses octopamine, the insect counterpart of vertebrate noradrenaline, as important signalling molecule (Kula-Eversole et al., 2010). Relations to or interactions with the PDF-system remain to be revealed.

3.4.2. PDF is a network-coordinator

With the generation of a null mutant for the *pdf* gene of the fruit fly, Renn et al. (1999) were able to functionally study the influence of this neuropeptide in detail and prove it to be a true clock output gene. Both *pdf* mutant flies and animals with ablated *pdf* neurons showed similar behavioural phenotypes when scoring the activity levels, indicating that *pdf* is the principal signalling molecule in these neurons. The mutant flies remained rhythmic in LD conditions, but lost rhythmicity after two days in DD; thus reflecting the gradual loss of pacemaker synchronisation by PDF (Renn et al., 1999). It is noteworthy that *Drosophila virilis* activity rhythms interestingly resemble those of *D. melanogaster pdf* null mutants. Additionally, *D. virilis* do not stain for PDF in the s-LNvs (Bahn et al., 2009), the major neurons to mediate PDF output regulation of locomotor behaviour (Park and Hall, 1998; Park et al., 2000). This led the authors to the promising hypothesis that different regulation of *pdf* transcription might be responsible for species-specific activity patterns (Bahn et al., 2009). Further experiments will need to clarify this possibility.

A small fraction of mutant *D. melanogaster* that remained rhythmic during DD indicates that there must be additional pacemaker neurons involved in regulating circadian behaviour (Renn et al., 1999). It has been proposed that the initial weak rhythmicity in the *pdf* null DD-flies is due to dampening PER oscillations in the DN1 neurons. Studying flies that lack the DN1 on the contrary revealed that their oscillations are dependent on PDF expression in the LNvs (Klarsfeld et al., 2004). The need of PDF for robust free-running rhythms is supported by a study of Peng et al., where it was shown that the LNvs alone are not sufficient to maintain circadian locomotor activity, but that the neuropeptide is additionally required for coordination of the circadian network (Peng et al., 2003). If the individual clock neurons are not synchronised, the overall result will be arrhythmia. The fact that both *pdf* null and ablated flies show similar phenotypes, underscores that PDF secretion is probably the most important function of the LNvs (Renn et al., 1999). It should nevertheless be noted that *pdf* expressing s-LNv neurons coexpress short neuropeptide F, which is diversely expressed in the fruit fly's brain (Johard et al., 2009; Kula-Eversole et al., 2010). Further study revealed that PDF is not required to maintain circadian protein oscillations, but that it is crucial to coordinate the phase and amplitude of circadian protein oscillations under constant conditions (DD) (Lin et al., 2004). There seems to be a hierarchical communication from the LNvs to the LNd, with PDF acting to delay phases in order to maintain synchrony. This action can probably molecularly be translated to the regulation of PER-entrance into the nucleus via kinases such as DOUBLETIME, SHAGGY and CALMODULIN KINASE II. Not only does PDF entrain the LNd neurons, it also feeds back to help synchronise the s-LNvs. If then all pacemaker cells generate in-phase, robust circadian PER-cycling, the overall generated rhythm will surpass a threshold that is needed for phenotypic day–night rhythmicity (Lin et al., 2004).

The network-coordinating action of PDF on the aMe neurons is related to GABAergic signalling in the cockroach. The effects of GABA and PDF on excised aMe revealed detailed organisation and function of the cockroach aMe. Ultradian firing aMe neurons are organised into phase-locked assemblies via GABAergic interneurons. Different assemblies have a stable phase difference. They can be synchronised to the same phase and period by PDF, thus generating resonance and producing a sufficient signal to cause postsynaptic cell firing. PDF acts inhibitory on GABAergic and other inhibitory interneurons, thereby activating the output to locomotor centres (Schneider and Stengl, 2005).

Recent findings show that PDF feedback on clock neurons influences their period in a dual way: it will lengthen the period of some clock neurons closely associated with the aMe, while that of others in the dorsal brain shortens with increasing PDF input. Using

D. melanogaster mutants that show gradually increasing l-LNv projections into the dorsal brain instead of the aMe, it could be shown that PDF causes the observed period alterations, because in *pdf* null background these changes disappeared. The neuropeptide therefore seems to act differently at different sites of the brain (Wülbeck et al., 2008). Further evidence for this hypothesis was gathered when studying the effects of PDF on specific subgroups of clock neurons. For some clock neurons, PDF exerts a period lengthening and synchronisation function. In others, it is not needed for these functions, but has a strong period influence that can either be lengthening, shortening or both depending on PDF concentration. In the latter case, neuropeptide F may counteract PDF effects. These neurons with multicomponent rhythms also appear to depend on environmental signals. This is interesting because it suggests a critical role for PDF in the coordination of the entire clock network, where regulation of phases of different neuron groups represents a natural function in seasonal adaptation (Yoshii et al., 2009). Activation of the PDF receptor similarly accelerates certain clock neurons, while decelerating others (Choi et al., 2009).

Another argument for differential regulation with regard to PDF function can be found in flies with altered *shibire*, a homologue of *dynammin*. On one hand, these animals display altered behavioural period, but only when PDF is also present. This indicates a PDF-dependent influence on clock output pathways. On the other hand, SHIBIRE can alter the s-LNv molecular clock, even in a *pdf* null background. Therefore, this effect is a consequence of a novel, PDF-independent pathway (Kilman et al., 2009).

3.4.3. Possible role in circadian rhythm plasticity

Social insects show plasticity of circadian rhythms among colony members, related to their specific tasks. *Bombus terrestris* workers are divided in large and small individuals related to their colony activities. Large bumblebees have daytime activities outside the colony and show strong circadian rhythmicity, whereas smaller bees usually stay in the nest to tend brood arrhythmically around the clock. In general, large *B. terrestris* have one additional PDH-ir neuron per brain hemisphere compared to smaller bees. The origin of this difference is currently not known, but may be due to developmental processes in pre-adult stages. The change in PDH-ir neuron number may affect the observed rhythm differences between large and small bumblebees (Weiss et al., 2009).

3.4.4. PDF action under temperature cycles

Although circadian clock function is mostly studied with regard to changing light conditions, temperature cycles can drive circadian rhythmicity under LL as well as DD conditions; this is driven by other neurons than *pdf* expressing cells (Yoshii et al., 2005; Glaser and Stanewsky, 2005, 2007; Sehadová et al., 2009 and references therein). In fact, a combination of differences in light intensity and temperature may result in altered behaviour dictated by the same cells. For example, DN1 neurons can promote both morning and evening activity, depending on these environmental factors (Zhang et al., 2010b). In absence of LD cycles, a subset of the dorsal neurons, the DN2 neurons, seem to entrain the entire network. While under LD cycles, the PDF positive LN phase the DN2 groups by signalling light information via PDF/PDFR, the hierarchy is conversed under temperature cycles in constant darkness (Picot et al., 2009). The molecular signalling mechanisms are yet to be clarified.

3.4.5. Sexual dimorphism and multiple oscillators

With the determination of the *D. melanogaster* PDF sequence, Park and Hall were also able to show that *pdf* is a sexual dimorphic gene, being more abundant in male flies (Park and Hall, 1998). The functional significance of this difference is yet unknown, but it is remarkable that PDF neurons are crucial for maintenance of male

courtship behaviour (Fujii and Amrein, 2010). Also, male *D. melanogaster* show an earlier morning activity peak than females do (Helfrich-Förster et al., 2000; Helfrich-Förster, 2000). This corresponds well to the hypothesis that PDF regulates the morning activity peak, but only partially the evening peak (Rieger et al., 2006). The morning oscillator alone is sufficient to drive 24 h rhythms in DD (Grima et al., 2004). The PDF LNVs exert a dominant function in timing the 24 h rhythm and impose their rhythm on other brain pacemaker cells (Blanchardon et al., 2001). Using a novel electrochemical technique that silences PDF neuron electrical activity by the targeted expression of a K⁺ channel, this hypothesis could be proven (Wu et al., 2008b). The transition of light to dark conditions does not require the LNVs (Dushay et al., 1989; Hardin et al., 1992), but is driven by the LNDs (Grima et al., 2004), the fifth *pdf*-negative LNV and some of the DN (Stoleru et al., 2004; Rieger et al., 2006). There is, however, timing information exchange between LNVs and LNDs (Stoleru et al., 2005); with the LNVs being the main pacemakers, contributing to both morning and evening peaks (Taghert and Shafer, 2006). PDF signals from the LNV morning cells affect the evening cells via PDFR activation (Choi et al., 2009; Lear et al., 2009).

Opposed to LNV action in DD, these cells cannot drive activity rhythms in constant light conditions, which are present only if there is also reduced CRYPTOCHROME (CRY) function (Picot et al., 2007). The *D. melanogaster* evening oscillator seems to receive light information from both the visual system and via CRY. PDF secreted by the L-LNVs is capable of phasing the evening oscillator molecular clock only when the CRY-function is absent or reduced; which is relevant on winter days. This PDF-function does not act via transcriptional control of *per* and is considered clock-independent. If both *pdf* and *cry* are absent, flies show no evening activity peak at all in LD and reverse the peak of their freerun in LL from the evening to the morning. This is thought to be a consequence of antiphase PER cycling in the evening oscillator, but not in the morning oscillator (Cusumano et al., 2009). Similar results were obtained for the relation between *pdf* and *cry*, additionally indicating that CRY can gate the PDF signal (Zhang et al., 2009). The division between morning and evening cells might be far more complex than previously thought, with a possibility of redundancy in the signalling system. The interactions of PDF and other signalling systems need further dissection (Lear et al., 2009).

3.4.6. Influence on peripheral oscillators

Besides the central brain molecular clock, several peripheral oscillators are involved in different circadian rhythms of the fruit fly. Ectopic expression of *pdf* not only has an influence on activity, but also affects eclosion (Helfrich-Förster et al., 2000). Deciphering eclosion timing revealed the first peripheral fly oscillator under control of the accessory medulla clock. Gating of eclosion timing to the hours around dawn is regulated by a clock in the prothoracic gland, which in turn requires input from the fly's brain clock to maintain eclosion rhythms. This input signal is most likely PDF derived from the lateral neurons (Myers et al., 2003).

The fly's eye oscillator, the circadian rhythm in olfactory responses as well as the circadian egg-laying rhythm are all independent of *pdf* expression (Tanoue et al., 2004; Zhou et al., 2005; Howlader et al., 2006).

3.4.7. Relation to the reproductive system

Hamanaka et al. revealed that induction of reproductive diapause of female blowflies is regulated by PDF, thereby confining a reproductive function to the neuropeptide. Pars lateralis neurons induce reproductive diapause under short-day and low-temperature conditions. They receive synaptic input from PDFMe neurons, where PDF was observed in dense core vesicles, but not in clear

synaptic vesicles. It is proposed that PDFMe neurons convey time or photoperiod information to the pars lateralis neurons (Hamanaka et al., 2005).

Additionally, circadian inputs to neurons producing prothoracicotrophic hormone (PTTH) have been suggested for many insects. PTTH is related to the growth factor superfamily and is in all insects responsible for modulation of ecdysteroid synthesis by the prothoracic glands. It has been shown that *R. prolixus* PTTH release is under circadian control, and neuroanatomical data proves that PTTH neurons are in tight association with PDF-positive neurons. This association has been observed in larvae, continues in the animal's adult life and is even strengthened by additional PTTH and PDF neurons, but the presumed function of PDF as regulator of steroidogenesis in adults is yet to be elucidated (Vafopoulou et al., 2007, 2010).

Observed links between circadian locomotor activity and the endocrine system (Persson et al., 2001; Silvegren et al., 2005) ask for further study. Throughout development of the German cockroach, the circadian clock is completed in different stages, indicated by differential presence of PDF cells and projections. In nymphs of this species, some of the PDF-positive neurons also stain for corazonin, and *vice versa*. Interestingly, corazonin exerts pigment-dispersing actions in crayfish integument (Porrás et al., 2003). It is suggested that PDF and corazonin work together in some of the output pathways of the circadian clock, possibly bridging the timing mechanism with the endocrine regulation of locomotion (Yang et al., 2009). It is noteworthy that in adult cockroaches, corazonin does not colocalise with, but is in close vicinity to PDF (Wen and Lee, 2008). Corazonin function seems less important in adults than it may be in nymphs (Yang et al., 2009), but there is a growing interest in this peptide as putative "alternating partner" for PDF in the regulation of clock-related functions (Boerjan et al., 2009).

3.4.8. Geotaxis

In *D. melanogaster*, the *pdf* gene has been shown to influence geotactic behaviour. Interestingly, this is also true for another clock gene: *cryptochrome*. The influence of both genes on geotaxis is nevertheless clock independent, since deletion of the *period* gene shows no such effect. While *pdf* is one of the multiple genes contributing to the overall geotactic phenotype, it is noteworthy that for this gene the effect on geotaxis is sex-related. In general, *pdf* null mutants show negative geotactic behaviour, meaning that they will fly against gravity (upwards) more than wild type flies do. Adding gradually more copies of the *pdf* gene was related to a trend downward in geotactic behaviour for both sexes; whereas deletion of the *pdf* gene did not influence male geotactic behaviour, but significantly decreased geotactic behaviour of female flies (Toma et al., 2002). It is known that the LNVs mediate cocaine induced loss of negative geotaxis in adult flies, but not via PDF, indicating that another LNV neurotransmitter participates in this phenomenon (Tsai et al., 2004).

3.4.9. Systemic action

Over the years, research has focused on PDF actions in the central brain. Based on localisation patterns, a function for PDF in the regulation of both the blowfly's foregut and hindgut elements was proposed (Nässel et al., 1993), but we found no further records investigating this possibility. Also, an elaborate study of locust PDFAb neurons provides supporting evidence for endocrine functions of PDF outside the central nervous system, as the peptide was found to be present in the hemolymph. In the locust abdomen, PDF could modulate the muscle control of the genital organs (Persson et al., 2001). For certain, there are some interesting research issues waiting at the level of systemic PDF action.

3.5. A PDF receptor in insects

A crucial breakthrough for functional PDF research was achieved when three groups simultaneously reported on the identification of the PDF receptor in the fruit fly *D. melanogaster*, which resulted in three names: *pdfR*, *han* (Korean for 'cold') and *gop* (groom of PDF) – we will further use *pdfR*. Mertens et al. found that PDFR is a class B G-protein coupled receptor (GPCR) that uses Gs to couple to adenylate cyclase and regulate cAMP levels. They could also show that *neurofibromatosis1* (*nf1*), which acts downstream of *pdf*, increases response and sensitivity of the receptor. This led to the suggestion that NF1 might couple the receptor to adenylate cyclase, similar to its mammalian function (Mertens et al., 2005).

PDFR displays sequence homology to both mammalian VPAC₂ and calcitonin receptors. Calcitonin and *D. melanogaster* calcitonin-like diuretic hormone 31 (DH31) could also elicit a response of PDFR, albeit to a lesser extent (Mertens et al., 2005). Interestingly, the *in vivo* s-LNV response to DH31 seems to partly depend on the PDFR because the molecule uses both PDFR and a specific DH31 receptor for signalling (Shafer et al., 2008).

As there were some differences in localisation patterns described in the first studies, additional experiments were needed for detailed information. Later expression experiments confined PDFR to all s-LNVs, all LNDs, to a lesser extent in the l-LNVs, in approximately half of the DN1, two DN3 and sometimes in one of the two DN2 (Shafer et al., 2008; Lear et al., 2009). About thirteen neuron pairs along the VNC of third instar larvae produced *pdfR*, but no embryonic expression was observed (Hyun et al., 2005). About one fifth of all *pdfR* product is present in the body; the rest is confined to the brain (Lear et al., 2005b). No diurnal *pdfR* RNA rhythm has been observed, suggesting that *pdfR* is regulated at steady-state level by *per* (Mertens et al., 2005; Lear et al., 2005b). Because even the above reported studies are thought to produce incomplete data, in a recent study, transgene flies were generated, thus enabling PDFR localisation based on the gene's own regulatory elements. A most interesting finding emerging from this study is the detection of *pdfR* expression in non-clock brain cells and in the visual system (Im and Taghert, 2010). These data suggest that PDF may use non-neuronal cells to regulate the visual input to the circadian system, a suggestion that has also been made for the cricket (Saifullah and Tomioka, 2003).

A role as local neurohormone has been suggested since most *pdf* and *pdfR* cells do not seem to be synaptically connected (Lear et al., 2005b). *In vivo* cAMP imaging revealed that nearly all pacemaker cells, whether they express PDF or not, respond to PDF. The l-LNVs are unique in that they display no or minor responses to PDF. This technique furthermore proved that the effect of PDF on the s-LNVs is indeed direct and acts through PDFR. Similarly, the effects of PDF on non-PDF expressing pacemaker cells are likely direct (Shafer et al., 2008).

PdfR mutants phenotypically copy *pdf* null mutants: they show aberrant behavioural rhythmicity and have a severe negative geotaxis phenotype (Mertens et al., 2005). However, the absence of the morning peak does not seem to be stably associated with *pdfR* mutations, whereas an advanced evening activity is common to both *pdf* and *pdfR* mutant flies (Im and Taghert, 2010). The *pdf* null mutant arrhythmicity in DD conditions could in a recent study be rescued by using membrane-anchored PDF molecules, most likely as a result of receptor activation in the dorsal evening cells (Choi et al., 2009). *PdfR* mutants show a phase advance of PER oscillation (Lear et al., 2005b) similar to *pdf* null mutants (Lin et al., 2004). The PDF signal can be prolonged by blocking SHIBIRE, a dynamin homologue that interferes with PDFR-mediated endocytosis. This receptor-mediated endocytosis influences the period of behavioural rhythms (Wülbeck et al., 2009).

It can be stated that since there is *pdfR* expression on the clock neurons themselves, *pdf* must feed back on these neurons (Im and

Taghert, 2010). The mode of action might be via diffusion or indirect neuronal connections, as most pacemaker neurons contain only low receptor amounts (Mertens et al., 2005). The primary synchronisation role of PDF is furthermore confirmed by an experiment wherein reasonably normal activity patterns could be restored in *D. melanogaster* when expressing *pdfR* in all clock neurons, but not in other *pdfR*-expressing neurons (Hyun et al., 2005). Looking in further detail, PDFR activation in the dorsal clock neurons appears important for rhythm generation, whereas PDF feedback to the LNVs has but a mild influence on circadian behaviour (Choi et al., 2009).

3.6. Control of the PDF signal

The PDF signal is regulated at different levels, resulting in a plethora of activating and inhibiting influences contributing to the overall PDF level. There is a general interplay between purely molecular and electrical signals.

3.6.1. Molecular control

Pdf-expression in *D. melanogaster* is stimulated by CLOCK (CLK) and posttranscriptionally inhibited by VRILLE (VRI) (Table 4). VRI, a bZIP transcription factor with a developmental role (George and Terracol, 1997), is itself a clock-controlled gene in the fruit fly (Blau and Young, 1999). It appears that CLK is only required for *pdf* expression in the larval LN and in the s-LNVs, since a mutation in the *clk* gene does not affect the *pdf* expression in l-LNVs and PDFAB neurons (Park et al., 2000). The heterodimerous partner of CLK, CYCLE, exerts similar but less severe effects on *pdf* expression. Mutations in both genes are additionally accompanied by developmental defects of PDF neurons, yet *clock* and *cycle* seem to influence *pdf* in an indirect fashion (Park et al., 2000). Interestingly, PDF peptide levels are influenced by mutations in either *period* or *timeless*, demonstrating a posttranslational, circadian regulation: PER and TIM could establish a temporal release gating for PDF, acting – in contrast to other *per* and *tim* phenotypes – in a counterproductive way (Park et al., 2000) (Table 4).

PDP1ε is one of the seven isoforms of the *D. melanogaster* *pdp1* gene and is thought to be involved in both the central timing mechanism of the circadian clock and in output. PDP1ε and VRI have opposite effects on *clock* transcription, thereby indirectly influencing *pdf* transcription (Table 4). Restoring *clk* expression in a *pdp1ε* mutant background remarkably is unable to restore either *pdf* expression or behavioural rhythms, indicating that the effect of PDP1ε on PDF is not strictly through *clk* (Zheng et al., 2009).

In a study of the *neurofibromatosis-1* gene it is suggested that binding of PDF to its receptor may influence the Ras/MAPK pathway that is also regulated by neurofibromin, the *nf1* gene product. MAPK activity cycles in the dorsal brain and participates in behavioural rhythmicity (Williams et al., 2001).

Looking upstream of *pdf*, there is some evidence that the *disco* gene controls the expression of clock genes: whereas it is usually assumed that the *disco*¹ mutation eliminates the LN from the brain (Helfrich-Förster and Homberg, 1993), PDF- and PER-negative lateral neurons have been detected in *disco*¹ mutants (Blanchardon et al., 2001). The earlier results are based on the use of PDF as a marker for the presence of the LN. Using a *gal1118*-driven GFP marker, it could be shown that most *disco*¹ mutants still possess at least one LN, but do not stain for PDF or PER (Blanchardon et al., 2001).

The LARK mRNA-binding protein mediates the temporal regulation of eclosion (Newby and Jackson, 1993) and has a general role in neuron excitability. Interestingly, when overexpressed only in the PDF-positive LNVs, it will act different in small and large neuron subgroups, and also in relation to the timing of overexpression. Overexpression during development influences the morphology of the s-LNVs, but not the l-LNVs: under

Table 4

Intracellular molecular interactions regulating PDF levels ($\pm$: in- or decrease of *pdf* expression or PDF presence). Indirect actions of VRI and PDP-1 ϵ requiring *clk* have been described. The mode of operation of other actions is yet to be elucidated.

	via <i>clk</i>	Not via <i>clk</i>	
		Transcriptional	Posttranscriptional
CLK		+	
CYC		+	
VRI	–		–
PDP1 ϵ	+	Other than via <i>clk</i>	
PER			Influence on PDF cycling
TIM			Influence on PDF cycling

these conditions, the s-LNV projections fail to reach their targets or the entire s-LNVs may even be eliminated. On the other hand, when overexpression is present in adult life, neuron morphology remains unaltered. Adult PER cycling is furthermore perturbed only in the l-LNVs, whereas PDP1 ϵ cycling is altered in the s-LNVs (Huang et al., 2009). The observed locomotor phenotype may indicate aberrant PDF function, but this remains to be investigated.

3.6.2. Electrical activity

While the LNVs synchronise oscillations in downstream neurons, their proper membrane excitability is regulated upstream and gates PDF release. As a consequence, excitability of the LNVs is critical for coherent rhythms; revealing a combined electrical and neuropeptide regulation of the clock neurons (Nitabach et al., 2006; Wu et al., 2008a). In the housefly, for example, PDF release is triggered by depolarisation (Miśkiewicz et al., 2008).

A potential candidate for electrical upstream PDF neuron regulation is SHAW, an endogenous neuronal potassium channel that regulates the resting membrane potential. SHAW probably modulates the electrical activity of clock neurons, amongst which the DN seem the major target. Changing SHAW levels in the DN proved to have the greatest influence on both locomotor activity as well as rhythmic PDF accumulation in the LNV terminals. Thus in turn, the DNs affect the rhythmic release of PDF (Hodge and Stanewsky, 2008). It should however be noted that many *shaw*-expressing cells also contain the PDF receptor; the molecular action of SHAW and its relation to PDF need further clarification.

Another candidate for PDF release regulation is the ion channel NARROW ABDOMEN (NA). *Na* mutants show elevated PDF levels in the dorsal projections of the s-LNVs, even under conditions in which PER is largely unaffected, indicating its output function (Lear et al., 2005a). A recent study also revealed the necessity of *na* expression in a subset of the posterior dorsal neurons 1 (DN1p) for mediating photic responses and morning anticipatory behaviour. As *pdf* receptor expression in these neurons is equally required for this behaviour, it has been proposed that the DN1p neurons comprise the nexus of Pdf and photic (NA) signalling (Zhang et al., 2010a).

In addition, both the *D. melanogaster* *slowpoke* and *slowpoke binding protein* (*slob*) genes have been associated with PDF. SLOB is a component of a dynamic protein complex that modulates the activity of the SLOWPOKE Ca²⁺-activated K⁺-channel and thus affects membrane excitability. As cyclic release of neuropeptides may be a consequence of daily changes in membrane potential and SLOB oscillates in photoreceptor nuclei and pars intercerebralis neuron cytoplasm, this protein is thought to be involved in the circadian system. In *pdf* null mutant flies, SLOB expression is furthermore reduced in pars intercerebralis neurons in the vicinity of the normally PDF-filled s-LNV terminals, implicating PDF as an upstream activator. PDF-mediated SLOB regulation probably acts via dorsal action of the l-LNVs (Jaramillo et al., 2004). Interestingly, the *slowpoke* mutant displays arrhythmic activity behaviour, probably as a result of aberrant PDF accumulation or release in the dorsal protocerebrum (Fernández et al., 2007).

There are some interesting aspects on PDF action when studying hyperexcitable versus silenced LNVs. Targeted expression of a voltage-gated sodium channel (NaChBac) in the LNVs of *D. melanogaster* renders them hyperexcitable and results in apparent asynchronous molecular oscillations after transfer to DD, reminiscent of PDF action (Nitabach et al., 2006). Long term DD observations of flies with such perturbed LNV activity contrarily showed that the circadian circuit evolved towards a new rhythmic balance. Not only PDF-dependent, but also PDF-independent factors thereby contribute to a circadian circuit-wide regulation of molecular oscillations and electrical activity of the LNVs, coordinating the different outputs (Sheeba et al., 2008c). When the LNVs are conversely silenced by targeted expression of a potassium channel, flies phenocopy the *pdf* null mutant flies both in behaviour and molecular oscillations in non-LNV clock neurons, except for a complete abolition of free-running oscillations in the LNVs themselves. This suggests that the residual LNV oscillations in *pdf* null mutants have no functional effect; therefore the LNVs do normally not use PDF-independent signalling mechanisms (Wu et al., 2008a), meaning that this signal must be derived from other cells.

3.6.3. Release and inactivation

The tetanus-toxin light chain (TeTxLC) blocks chemical synaptic transmission by targeting synaptobrevin, a molecule co-responsible for membrane fusion in exocytosis of secretory vesicles. When TeTxLC is expressed under control of the *pdf* promoter, the behavioural activity pattern of flies showed little changes (Kaneko et al., 2000). Considering the evidence for the importance of the *pdf* expressing cells in rhythmic behaviour (Helfrich-Förster, 1998; Stanewsky et al., 1998; Renn et al., 1999), it is most likely that the TeTxLC does not sufficiently block *pdf* release in *D. melanogaster* (Kaneko et al., 2000); meaning that PDF transmission does not use synaptobrevin-dependent signalling (Blanchardon et al., 2001).

After its release from the presynaptic site and action on target neurons, PDF is rendered inactive through hydrolysis by the extracellular peptidase neprilysin. The *D. melanogaster* genome harbours 25 neprilysin-like molecules, it still needs to be revealed which one(s) is (are) responsible for this action (Isaac et al., 2007).

4. Pigment dispersing factor in nematodes

4.1. The discovery and diversity of pigment dispersing factor peptides in nematodes

Caenorhabditis elegans is the best studied representative among nematodes. *C. elegans* exhibits a clear circadian rhythm in activity and in resistance to osmotic and oxidative stress (Saigusa et al., 2002; Hasegawa et al., 2005; Simonetta and Golombek, 2007; Simonetta et al., 2008; Simonetta et al., 2009). The *C. elegans* genome contains homologues of many of the central regulators of circadian timing in *D. melanogaster*, like *lin-42* (~period), *tim-1* (~timeless), *kin-20* (~doubletime), *sma-9* (~clock), *aha-1* (~cycle) and *atf-2* (~vrille). Most of these genes, however, are involved in the timing of development. The general view stipulates that the core of the circadian clock in *C. elegans* is composed differently than that of insects (Jeon et al., 1999; Chan et al., 2003; Banerjee et al., 2005; Hasegawa et al., 2005).

Our group recently added PDF to the list of clock homologues by means of a reverse pharmacological screen. The *C. elegans* PDF system consists of three different PDF peptides called PDF-1a, PDF-1b and PDF-2, and three PDF receptors called PDFR-1a, PDFR-1b and PDFR-1c. All three *C. elegans* PDF peptides were able to activate each of the three *C. elegans* receptors in a dose-dependent manner, albeit with different affinities (Janssen et al., 2008, 2009).

Similarly to crustacean PDH and insect PDF, originates the nematode PDF peptide from a larger precursor molecule consisting

of a signal peptide, a linking peptide and the mature PDF flanked by dibasic cleavage sites.

A tBLASTn analysis of the *Ce*-PDF-1 and *Ce*-PDF-2 precursors revealed an extremely strong conservation within the phylum of nematodes wherein four (Rhabditina, Tylenchina, Spirurina and Dorylaimia) of the five nematode clades contain species that have clear *pdf* orthologues (Table 5) (Parkinson et al., 2004; Janssen et al., 2009). Based on amino acid sequence, nematode PDF-like peptides are more related to β -PDH than to α -PDH. They are however more distantly related to β -PDH than the insect PDF peptides and contain 20–22 amino acids instead of 18.

4.2. Pigment dispersing factor localisation

Spatial expression patterns of *C. elegans* PDF were examined using translational green fluorescent protein (GFP) reporter constructs. The *C. elegans* PDF-1 precursor gene (encoding both PDF-1a and PDF-1b peptides) is expressed throughout postembryonic life in interneurons, (chemo)sensory neurons and motor neurons. Also a few non-neuronal tissues like rectal gland cells and intestinal rectal valve cells express these peptides. *Pdf-2* expression was similar and could be observed during postembryonic life in interneurons, (chemo)sensory neurons, motor neurons and also

Table 5

Amino acid sequence alignment of nematode PDF-like peptides. Identical amino acids are highlighted in black, conserved amino acids in dark gray and similar amino acids in light gray.

Species	Peptide sequence	Reference
Nematode PDF-1a		
Caenorhabditis elegans	SNAELINGLIGMDLGKLSAVamide	(Janssen et al., 2008; Janssen et al., 2009)
Caenorhabditis briggsae*	SNAELINGLIGMDLGKLSAVamide	
Caenorhabditis remanei*	SNAELINGLIGMDLGKLSAVamide	
Caenorhabditis brenneri*	SNAELINGLIGMDLGKLSAVamide	
Caenorhabditis japonica*	SNAELINGLIGMDLGKLSAVamide	
Heterorhabditis bacteriophora*	SNAELINGLIGMDLGKLSAVamide	
Pristionchus pacificus*	SNAELINGLIGMDLGKLSAVamide	
Ancylostoma caninum*	SNAELINGLIGMDLGKLSAVamide	
Ancylostoma ceylanicum*	SNAELINGLIGMDLGKLSAVamide	
Necator americanus*	SNAELINGLIGMDLGKLSAVamide	
Teladorsagia circumcincta*	SNAELINGLIGMDLGKLSAVamide	
Dictyocaulus viviparus*	SNAELINGLIGMELGKLSAVamide	
Strongyloides stercoralis*	SNAELINGLIGMDLGKLSAVamide	
Bursaphelenchus xylophilus*	SNAELINGLIGMDLNLKLSAVamide	
Ascaris suum*	SNAELINGLIGMDLNLKLSAVamide	
Ditylenchus africanus*	SNAELINGLIGMDLNLKLSAVamide	
Nematode PDF-1b		
Caenorhabditis elegans	SNAELINGLIGMNLNKLSSAamide	(Janssen et al., 2008; Janssen et al., 2009)
Caenorhabditis briggsae*	SNAELINGLIGMNLNKLSSAamide	
Caenorhabditis remanei*	SNAELINGLIGMNLNKLSSAamide	
Caenorhabditis brenneri*	SNAELINGLIGMNLNKLSSAamide	
Caenorhabditis japonica*	SNAELINGLIGMNLNKLSSAamide	
Heterorhabditis bacteriophora*	SNAELINGLIGMNLNKLSSAamide	
Pristionchus pacificus*	SNAELINGLIGMNLNKLSSAamide	
Ancylostoma caninum*	SNAELINGLIGMNLNKLSSAamide	
Ancylostoma ceylanicum*	SNAELINGLIGMNLNKLSSAamide	
Necator americanus*	SNAELINGLIGMNLNKLSSAamide	
Teladorsagia circumcincta*	SNAELINGLIGMNLNKLSSAamide	
Dictyocaulus viviparus*	SNAELINGLIGMNLNKLSSAamide	
Strongyloides stercoralis*	SNAELINGLIGMDLNLKLSAamide	
Bursaphelenchus xylophilus*	SNAELINGLIGMDLNLKLSAamide	
Ascaris suum*	SNAELINGLIGMNLNKLSSAamide	
Onchocerca volvulus*	SNAELINGLIGMDLNLKLSAamide	
Trichinella spiralis*	SNAELINGLIGMDLNLKLSAamide	
Nematode PDF-2		
Caenorhabditis elegans	NNAEVVNHLKKNFGALDRLGDVamide	(Janssen et al., 2008; Janssen et al., 2009)
Caenorhabditis ramenei*	NNAEVVNHLKKNFGALDRLGDVamide	
Caenorhabditis. brenneri*	NNAEVVNHLKKNFGALDRLGDVamide	(Husson et al., 2009)
Caenorhabditis. japonica*	NNAEVVNHLKKNFGALDRLGDVamide	
Caenorhabditis briggsae	NNAEVVNHLKKNFGTLDRLGDVamide	
Ascaris suum*	NNAEVVNHLKKNFGALDRLGDVamide	
Bursaphelenchus xylophilus*	NNAEVVNHLKKNFGTLDRLGDVamide	
Necator americanus*	NNAEVVNHLKKNFGTLDRLGDVamide	
Ancylostoma caninum*	NNAEVVNHLKKNFGTLDRLGDVamide	
Ostertagia ostertagi*	NNAEVVNHLKKNFGTLDRLGDVamide	
Ancylostoma ceylanicum*	NNAEVVNHLKKNFGTLDRLGDVamide	
Teladorsagia circumcincta*	NNAEVVNHLKKNFGTLDRLGDVamide	
Nippostrongylus brasiliensis*	NNAEVVNHLKKNFGTLDRLGDVamide	
Brugia malayi*	NNAEVVNHLKKNLNEVDLLEGAamide	
Meloidogyne javanica*	NNAEVVNHLKKNFASINRLGDAamide	
Meloidogyne incognita*	NNAEVVNHLKKNFASINRLGDAamide	
Meloidogyne chitwoodi*	NNAEVVNHLKKNFASINRLGDAamide	
Meloidogyne hapla*	NNAEVVNHLKKNFASINRLGDAamide	

* Amino acid sequence derived from tBLASTn (ClustalW2 and BOXSHADE 3.21).

in rectal gland cells, intestine-rectal valve cells and three additional posterior arcade cells in the head that line the extreme inside edge of the buccal cavity. Apart from GFP fluorescence in somata, also several dendrites and axons showed *pdf* expression indicating that the PDF peptides fulfil a possible role in neuromodulation and/or transmission (Janssen et al., 2009).

4.3. Function and regulation

Almost all PDF neurons in *C. elegans* play a role in the sensing and integration of environmental stimuli such as mechanosensation, oxygen sensing and chemosensation or in the control of locomotion (Janssen et al., 2009). Mutants lacking PDF-1a and b display a severely altered locomotor behaviour manifested in a decreased moving speed, an increased reversal frequency and an increased frequency of directional changes. Overexpression of *pdf-2* phenocopies the locomotor defects of the *pdf-1* deletion mutant, suggesting that they elicit opposite effects on locomotion (Janssen et al., 2008). The individual PDF peptides do not seem to influence each others expression levels (Janssen et al., 2009). Although *pdf-1* mutants show an obvious locomotor phenotype, it has so far not been demonstrated that the PDF peptides are also involved in the regulation of circadian activity. Analogous to the *D. melanogaster* PDF system, *C. elegans* shows no daily variations in *pdf* (*pdf-1* and *pdf-2*) gene expression, as determined by RT-PCR (Janssen et al., 2009). Whether or not PDF levels fluctuate at protein level, has not yet been investigated. *C. elegans pdf* expression seems to be positively regulated at the mRNA level by two clock related proteins, i.e. ATF-2 (~*D. melanogaster* VRILLE) and CES-2 (~*D. melanogaster* PAR domain protein 1; PDP1ε) (Janssen et al., 2009). *Atf-2* in *ces-2* both encode bZIP transcription factors which have been suggested to regulate developmental cell fate in a temporal and spatial way (Wang et al., 2006; Hatzold and Conradt, 2008). In yeast two-hybrid experiments, ATF-2 and CES-2 were able to physically interact and form functional heterodimers that regulate the temporal transcription of certain genes e.g. *lin-48*. As described in Section 3.4.1, *D. melanogaster* VRILLE and PDP1ε regulate *pdf* expression respectively in a negative and positive way partially via CLOCK.

4.4. PDF receptors in nematodes

C. elegans contains three G protein-coupled PDF receptors which belong to the class B (secretin) type of GPCRs. These three receptors are in fact three isoforms encoded by the same gene, called *pdf-1*. PDF-2 is the most potent ligand of the PDF receptor isoforms a and b (Janssen et al., 2008). The *C. elegans* PDF receptors are expressed in several sensory- and motorneurons and in all body wall muscles (Janssen et al., 2008).

Class B GPCRs, including the *D. melanogaster* PDF receptor, typically signal through adenylate cyclase via $G_{\alpha_{s/i}}$ (Mertens et al., 2005). This is also the case for the *C. elegans* PDF receptor as was demonstrated in an *in vitro* CRE-luciferase reporter assay in HEK293 cells. Both PDFR-1a and PDFR-1b seem to signal through G_{α_s} , whereas PDFR-1c seems to signal through G_{α_i} (Janssen et al., 2008).

5. Homology to the vertebrate system

For insects, both the organisation of the circadian system in which PDF acts and the molecular actions of the neuropeptide are often compared to the vertebrate system (Fig. 6). As this comparison is not common for crustacean literature and too premature for nematodes, following introductory description will focus on the present knowledge for insect en vertebrate PDF signalling.

Fig. 6. Introductory scheme for comparison of core clock structure and PDF/VIP action between insects and vertebrates. (aMe, accessory medulla; SCN, suprachiasmatic nucleus; s/l-LNv, small/large ventrolateral neurons; VIP, vasoactive intestinal peptide) For detailed information: see text.

5.1. Structural organisation

The dorsal brain is an important region with regard to circadian timing in all insects. Although there are some insect species that seem to have the dorsal brain clock as master, the location of the master clock is generally the aMe. In mammals, the suprachiasmatic nuclei (SCN) of the hypothalamus comprise the master clock. The SCN and aMe can be compared on many levels. They are both in close vicinity to the optic system. The structural organisation of mammalian and insect central clocks is alike in that they both consist of a core that gets ipsilateral input and a shell that mainly contains projection neurons. This structural division of the SCN is based on a different cell architecture: the ventrolateral core consists of rather loosely associated, spherical neurons with many afferent fibers; while the dorsomedial shell comprises closely packed, elongated neurons with many efferent fibers. Although such split organisation is clearly visible for the well characterised cockroach aMe, it must be noted that the flies' aMe does not show an evident anatomical division. In the cockroach, the core contains tight neuropil knots named noduli, and the shell – or internodular neuropil – lies around and inbetween these structures. Similar to the SCN, the aMe core gets input from the ipsilateral photic system and the shell provides many efferent fibers. For mammals as well as for insects, the structural division is associated with different functionality for core and shell neurons. SCN core neurons contain, amongst others, vasoactive intestinal peptide (VIP) and calbindin. Calbindin neurons may be important in gating photic signals and are the possible counterparts of the PDF-positive l-LNv. Vasopressin is expressed in the shell neurons and acts as an output peptide. The *Drosophila* s-LNv are thought to be similar to the shell neurons. Additionally, both aMe and SCN communicate via diffusible substances and rely on glia cells for function (Helfrich-Förster, 2000; Helfrich-Förster, 2004).

5.2. VIP/VPAC₂ resembles PDF/PDFR

Whereas the PDFR shows sequence homology to VPAC₂, which is the mammalian receptor for VIP and PACAP; no sequence homologue for PDF exists in vertebrates (Mertens et al., 2005; Vosko et al., 2007). VIP is nevertheless the best candidate based on function. While PACAP is able to activate the *D. melanogaster* PDFR (Mertens et al., 2005), this has never been tested for VIP, nor are there any records of PDF action on VPAC₂.

Vasoactive intestinal peptide is synthesized in the ventrolateral region of the SCN, which is innervated by retinal cells. In contrast to the fly PDF/PDFR levels, both VIP and VPAC₂ mRNA show daily cycling (Vosko et al., 2007). VIP is involved in photic phase shifting of the SCN clock and phase-advances the release of arginine vasopressin (AVP), which is important in the output of the circadian clock. It also induces *Period* gene transcription (Harmar et al., 2002; Aton and Herzog, 2005). After release, both PDF and VIP are degraded by endopeptidases: neprilysin and neprilysin/kallikrein, respectively (Isaac et al., 2007).

In mammals, VPAC₂-cells include half of the VIPergic cells and almost all AVP-neurons of the dorsal SCN. VIP/VPAC₂ is needed for synchrony between SCN cells as well as within individual SCN neurons. The VIP/VPAC₂ signalling system is furthermore of major importance for coupling the oscillator to the environment. VIP may even feed back to regulate VIPergic cells, as they express VPAC₂. Both *VIP*^{−/−} and *Vipr2*^{−/−} mice show rhythm deficiencies that reflect their inability to maintain amplitude and circadian synchrony (Harmar et al., 2002; Aton and Herzog, 2005; Vosko et al., 2007). Similarly to PDF (Mertens et al., 2005), VIP binding leads to cAMP increases. VIP may modulate GABA-mediated transmission in the SCN in a cAMP-dependent way, thereby maintaining synchrony between SCN neurons (Vosko et al., 2007). For PDF, different effects on different clock neurons have been described; such a multiphasic clock organisation has also been reported for mammals (Yoshii et al., 2009).

VIP also seems to influence firing rates of a subpopulation of SCN neurons by regulating ion currents (Vosko et al., 2007). *Drosophila* and mammalian circadian neuron electrical characteristics closely resemble each other (Sheeba et al., 2008b); homologous ion channels have been reported to be involved in both systems (Hodge and Stanewsky, 2008).

Taking the focus away from locomotor activity, the insect PDF-positive clock cells as well as the mammalian SCN are both related to endocrine rhythms. VIP has been reported to play a role in the temporal regulation of gonadotropin-releasing hormone neurons and of the hypothalamic-pituitary-adrenal axis (Vafopoulou et al., 2007; Vosko et al., 2007).

6. Concluding remarks

In this review, we opted to cover the literature on the pigment dispersing-like molecular signalling systems. Pigment dispersing-like systems have been studied extensively over the past decades, and the rich variety of physiological processes in part regulated by these mechanisms is an example of the complexity of regulation and communication in living organisms.

Within the PDH and PDF precursor molecules the mature peptides are well conserved, whereas the PDH/PDF associated peptide which is also present in the precursor is poorly conserved and of unknown function. Most pigment dispersing-like precursors contain a signal peptide targeting them to the secretion pathway, but a nuclear localisation sequence has also been described.

Looking in detail, no general localisation patterns can be derived for these peptides, probably reflecting species-specific differences in function as well. The system is nevertheless invariably associated with the visual system, optimally localised

to modify visual information. Whereas the functions of pigment dispersing hormone-like molecules in the central nervous system are widely studied, the future is bound to reveal more information on the endocrine actions of this pleiotropic molecule. PDH has been shown to regulate crustacean colour changes and retinal sensitivity, thus influencing the darkening of the integument and acting as a natural pair of sunglasses for the eyes. In insects, the involvement of PDF in the circadian clock is intensively studied, as different research teams try to precisely describe the functions of the molecule herein. Also, non-circadian related processes like the peripheral oscillator regulating eclosion timing are influenced by PDF. It is also interesting that *D. melanogaster* PDF levels are a sex-dimorphic trait, possibly beneficial in reproductive behaviour. Associations with reproduction are present in different species, for example in the regulation of the induction of reproductive diapause of female blowflies. Other processes such as behavioural arousal and sleep, and geotaxis are also influenced by this neuropeptide. In nematodes, the only PDF-function so far described is related to locomotory behaviour.

Summarising the literature reported on one single molecule, reveals the diversity of amino acid sequences and functions among different species and also within species. Keeping this in mind, researchers from diverse fields can benefit from such knowledge, which may reveal molecular crossroads and interplays between studied processes. Further research in different species will not only provide deeper insight into the diversity of regulating and modulating systems, it will also reveal the evolution of this neuropeptidergic signalling system. The quest for knowledge continues.

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