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Metabolic inactivation of the circadian transmitter, pigment dispersing factor (PDF), by neprilysin-like peptidases in *Drosophila*

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Summary

Recent studies have firmly established pigment dispersing factor (PDF), a C-terminally amidated octodecapeptide, as a key neurotransmitter regulating rhythmic circadian locomotory behaviours in adult *Drosophila melanogaster*. The mechanisms by which PDF functions as a circadian peptide transmitter are not fully understood, however; in particular, nothing is known about the role of extracellular peptidases in terminating PDF signalling at synapses. In this study we show that PDF is susceptible to hydrolysis by neprilysin, an endopeptidase that is enriched in synaptic membranes of mammals and insects. Neprilysin cleaves PDF at the internal Ser7–Leu8 peptide bond to generate PDF1-7 and PDF8-18. Neither of these fragments were able to increase intracellular cAMP levels in HEK293 cells cotransfected with the *Drosophila* PDF receptor cDNA and a firefly luciferase reporter gene, confirming that such cleavage results in PDF inactivation. The Ser7–Leu8 peptide bond was also the principal cleavage site when PDF was incubated with membranes prepared from heads of adult *Drosophila*. This endopeptidase activity was inhibited by the neprilysin inhibitors phosphoramidon (IC_{50}, 0.15 μmol l^{-1}) and thiorphan (IC_{50}, 1.2 μmol l^{-1}). We propose that cleavage by a member of the *Drosophila* neprilysin family of endopeptidases is the most likely mechanism for inactivating synaptic PDF and that neprilysin might have an important role in regulating PDF signals within circadian neural circuits.

Key words: pigment dispersing factor, neprilysin, peptidase, neuropeptide, circadian rhythm.

Introduction

Drosophila melanogaster pigment dispersing factor (PDF; NSELINSLLSLPKNMNDAamide), a member of the arthropod pigment dispersing hormone/factor (PDH/PDF) family of peptides, is the solely identified chemical output of clock neurons in *Drosophila* (Helfrich-Forster, 2005; Taghert and Shafer, 2006). The importance of PDF as a neurotransmitter of the circadian clock was suggested by experiments employing immunocytochemistry, which showed that PDF is co-expressed with clock markers, specifically the *period* gene (Helfrich-Forster et al., 1998). Subsequent mosaic analysis has demonstrated that the LN_v (ventral lateral neurons) subset of pacemaker neurons, which express PDF, are required for rhythmic circadian locomotory behaviours in adult *Drosophila* (Helfrich-Forster et al., 1998). Confirmation that the PDF peptide is a circadian neurotransmitter came from studies of a *Drosophila* mutant line (pdf^{01}) lacking PDF (Peng et al., 2003; Renn et al., 1999) and flies misexpressing *pdf* (Helfrich-Forster et al., 2000). Wild-type *Drosophila* entrained to a 12 h:12 h light:dark cycle display robust rhythmic locomotory behaviour comprising crepuscular (dawn and evening) peaks of activity with periods of 'sleep' and 'rest' at night time and in the afternoon, respectively (Helfrich-Forster, 2005). Normal flies also show anticipation of morning and evening times, as illustrated by initiation of locomotory activity before the lights are switched on and off. This circadian rhythm is maintained when flies are transferred from a 12 h:12 h light:dark cycle to a constant dark environment. The locomotory activity of pdf^{01} null mutants is rhythmic under entraining schedules, but differs from wild-type behaviour in the loss of the anticipation of lights-on and in the advancement of the evening peak of activity (Renn et al., 1999). In constant darkness, pdf^{01} flies have reduced locomotory response to lights-on, and after 2 days become strongly arrhythmic (Renn et al., 1999). Further studies using the pdf^{01} mutants showed that PDF is required for synchronising a network of pacemaker cells in adult *Drosophila* (Lin et al., 2004; Peng et al., 2003). The discovery of the PDF-receptor gene (*PdfR*) and the behavioural analysis of *PdfR* mutants confirmed the critical role of PDF signalling in maintaining robust locomotory circadian rhythms (Hyun et al., 2005; Lear et al., 2005; Mertens et al., 2005).

There is much interest in understanding how PDF signals in a rhythmic manner and at what level this is regulated. Clock genes are known to control PDF expression, but this occurs at

a post-translational level, as neither PDF mRNA nor protein levels in the LN_v cell bodies cycle (Park and Hall, 1998; Park et al., 2000). Nevertheless, cycling of the mature PDF peptide has been observed in the terminals of the small LN_v cells of wild-type flies, suggesting that transport to and release from cell termini might provide a mechanism for the rhythmic action of PDF (Park et al., 2000). At present the importance of these observations is not entirely clear since it has been shown that some transgenic fly lines display wild-type-like locomotory rhythms, even in the absence of any cycling of PDF immunoreactivity at small LN_v cell termini (Kula et al., 2006).

Therefore questions remain as to the biological mechanism of how PDF functions as a circadian peptide transmitter in adult *Drosophila*. One aspect that has not received attention is the potential role of neuropeptidases in this mechanism, despite recent reports that several candidate peptidases either have been shown to cycle or are transcriptionally regulated by components of the circadian clock (McDonald and Rosbash, 2001). Such peptidases, typically with preferences for peptides of up to 4 kDa in size, are known to be important for terminating the signalling activity of neuropeptides at synapses. The best known of these is neprilysin (EC 3.4.24.11), a neutral zinc metallopeptidase with a broad tissue distribution in mammals (Turner, 2004; Turner et al., 2001). It was first described as a major protein of the brush border membrane of the kidney, but was re-discovered as the brain enzyme responsible for the degradation of enkephalin, hence its alternative name: enkephalinase (Matsas et al., 1985a). As a type II integral membrane protein, neprilysin has its active site facing the extracellular milieu whilst anchored by an N-terminal cytoplasmic peptide and a transmembrane domain (Turner et al., 2001). Enzymatic activity is optimal at neutral pH, and is inhibited by chelators of bivalent metal ions and by the *Actinomycetes* product, phosphoramidon, and additionally by synthetic inhibitors such as thiorphan (Turner, 2004). Although neprilysin cleaves a wide range of peptides, it shows specificity towards peptide bonds that use the amino group of an amino acid with a bulky hydrophobic side chain (Matsas et al., 1984; Turner, 2004). Neprilysin-like enzymes appear early in the evolution of peptidergic signalling in animal nervous systems, which is consistent with a key role in regulating neuropeptide activity (Turner et al., 2001). In insects, phosphoramidon-sensitive neprilysin activity is enriched in neuropil regions of the insect brain (*Schistocerca gregaria*, *Locusta migratoria* and *Leucophea maderae*) and in neural membranes (*Drosophila melanogaster*, *Musca domestica* and *Lymantria dispar*) (Isaac, 1988; Isaac et al., 2002; Lamango and Isaac, 1993; Masler et al., 1996).

We report that PDF is cleaved by human neprilysin at the Ser7–Leu8 peptide bond to generate two peptide fragments that do not elicit a response from cells expressing *PdfR*. Furthermore, we show that the major PDF-degrading activity of *Drosophila* head membranes cleaves PDF at the same peptide bond and that the hydrolysis is inhibited by the neprilysin inhibitors phosphoramidon and thiorphan. We suggest that a neprilysin-like neuropeptidase plays a role in the regulation of extracellular levels of PDF and could therefore be important for the functioning of PDF as a circadian neurotransmitter in *Drosophila*.

Materials and methods

Materials

PDF (NSELINSLLSLPKNMNDAamide) and recombinant human neprilysin were generous gifts from Professor D. Nässel, Department of Zoology, University of Stockholm, Stockholm, Sweden and Professor A. J. Turner, Faculty of Biological Sciences, University of Leeds, Leeds, UK. Insect peptides were either purchased from Bachem (UK) Ltd, St Helens, UK, or were custom-synthesised by Pepceuticals, Nottingham, UK. PDF1-7 and PDF8-18 were generated enzymatically by incubating PDF with recombinant human neprilysin. The two fragments were purified and quantified by high-performance liquid chromatography (HPLC) and their purity checked by MALDI-MS. Other chemicals, including thiorphan and phosphoramidon, were purchased from Sigma Chemical Co., Poole, Dorset, UK. HPLC solvents were from Rathburns, Walkerburn, Scotland, UK and the Jupiter 5 μ column (C18, 250 mm×4.5 mm i.d.) was purchased from Phenomenex, Macclesfield, UK.

Preparation of adult D. melanogaster head membranes

Drosophila melanogaster (*w1118* strain) were cultured on a standard oatmeal/molasses/agar diet at 25°C in a 12 h:12 h light:dark cycle. Heads (10 mg) were collected from adult *w1118* flies that had been frozen in liquid N_2 and were homogenised in 100 mmol l^{-1} Hepes buffer, 100 mmol l^{-1} NaCl (pH 7). The homogenate was centrifuged at 1000 ***g*** for 2 min and the pellet rehomogenised in 100 mmol l^{-1} Hepes buffer, 100 mmol l^{-1} NaCl (pH 7) and subjected to a second low-speed centrifugation. The previously saved supernatants were centrifuged at 30 000 ***g*** for 1 h in an Optima^{TM} MAX Beckman Coulter using a TLA110 rotor (Beckman Coulter, High Wycombe, Bucks, UK). The resulting membrane pellet was rehomogenised in a high-salt buffer (100 mmol l^{-1} Hepes buffer, 0.5 mol l^{-1} NaCl, pH 7) and membranes recovered by a second 30 000 ***g*** centrifugation step. The final pellet was resuspended in 100 mmol l^{-1} Hepes buffer, 100 mmol l^{-1} NaCl, pH 7, using a glass homogeniser to give a protein concentration of 350 μg ml^{-1}.

Hydrolysis of insect peptides

Peptide hydrolysis was performed by incubating 100 μmol l^{-1} peptide with either human neprilysin (1.25 ng of protein) or *D. melanogaster* head membranes (1 μg of protein) in a final volume of 20 μl of 100 mmol l^{-1} Hepes buffer, 100 mmol l^{-1} NaCl, pH 7 at 35°C. Enzyme activity was stopped by the addition of 5 μl of 8% (v/v) trifluoroacetic acid and the final volume was made up to 260 μl with 0.1% (v/v) trifluoroacetic acid. The parent peptide and peptide fragments generated by peptidase activity were resolved and quantified by reverse-phase HPLC using a Jupiter 5 μ column (C18, 250 mm×4.5 mm i.d.) and detection at 214 nm, as described previously (Isaac and Nässel, 2003). Rates of hydrolysis were determined by measuring the decline of the parent peptide at four time points over a 1 h period. For the assay for PDF-degrading activity of *D. melanogaster* head membranes, the rate of hydrolysis of PDF was determined by measuring the formation of PDF8-18 by HPLC (Isaac and Nässel, 2003). The production of PDF8-18 was linear with time when PDF

hydrolysis was less than 30%. The effects of peptidase inhibitors on enzyme activity were investigated by pre-incubating the enzyme with inhibitor for 10 min at 35°C prior to starting the reaction by the addition of peptide substrate. IC_{50} values were calculated using a curve-fitting algorithm (FigP, Biosoft, Cambridge, UK). A Voyager DE STR MALDI-TOF mass spectrometer (Applied Biosystems, Warrington, UK) was used to obtain mass spectra of PDF1-7 and PDF8-18, as described previously (Audsley and Weaver, 2007).

Functional PDF receptor assay

The relative potencies of PDF, PDF1-7 and PDF8-18 as activators of the *D. melanogaster* PDF receptor were determined essentially as described previously (Johnson et al., 2003; Meeusen et al., 2002) by using HEK293 cells co-transfected with the full-length cDNA for the PDF receptor gene (CG13758) (Mertens et al., 2005) and a firefly luciferase reporter gene construct that included a multimerised cAMP response element (CRE6x) ligated upstream from the reporter gene (George et al., 1998). Transfected HEK cells were incubated with the peptide for 5 h, then lysed, and a LucLite (Perkin Elmer, Waltham, MA, USA) was used to determine luciferase levels, which were read using a Victor Wallac2 Plate reader (Perkin Elmer). Three replicate wells from three independent transfections were used to generate EC_{50} values using nonlinear curve fitting (PRISM 3.0, GraphPad, San Diego, CA, USA).

Results and discussion

Cleavage of the Ser7-Leu8 peptide bond of PDF by neprilysin

Recombinant human neprilysin was used to determine the susceptibility of peptidase degradation for PDF and eight other insect neuropeptides. The results of this experiment showed a large variation in the rate of hydrolysis of individual peptide substrates (Fig. 1). Importantly, PDF was amongst the substrates most rapidly degraded by neprilysin, with a rate of hydrolysis comparable to that obtained with two members of the tachykinin family of neuropeptides (locustatachykinin-1 and locustatachykinin-2). The tachykinins are a family of evolutionarily conserved neuropeptides that are inactivated at synapses by neprilysin peptidases (Matsas et al., 1983; Matsas et al., 1985b; Barnes et al., 1993; Isaac and Nässel, 2003). Hydrolysis of PDF by recombinant neprilysin gave rise to two primary peptide fragments, which were subsequently isolated using HPLC (Fig. 2) and analysed by MALDI-MS. The early eluting peptide was identified as PDF1-7 ($[M+H]^+$, *m/z* 777; $[M+Na]^+$, *m/z* 799; $[M+K]^+$, *m/z* 815) and the later eluting fragment as PDF8-18 ($[M+H]^+$, *m/z* 1215), which identified the Ser7–Leu8 peptide bond as the primary site of neprilysin-induced cleavage.

Cleavage at the Ser7–Leu8 peptide bond of PDF by neprilysin is consistent with the strong preference of the peptidase for peptide bonds incorporating the amino group of

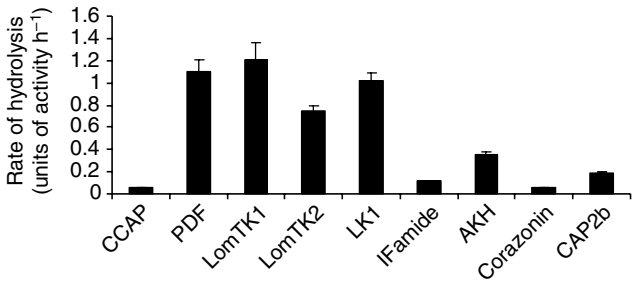

Fig. 1. Rates of hydrolysis of insect peptides by neprilysin. Rates were determined using HPLC to measure the linear decline in the peptide substrate during incubation with human neprilysin, as described in Materials and methods. Values are the means ± s.e.m. of at least three determinations. A unit of activity is 1 nmole of peptide hydrolysed per ng of neprilysin.

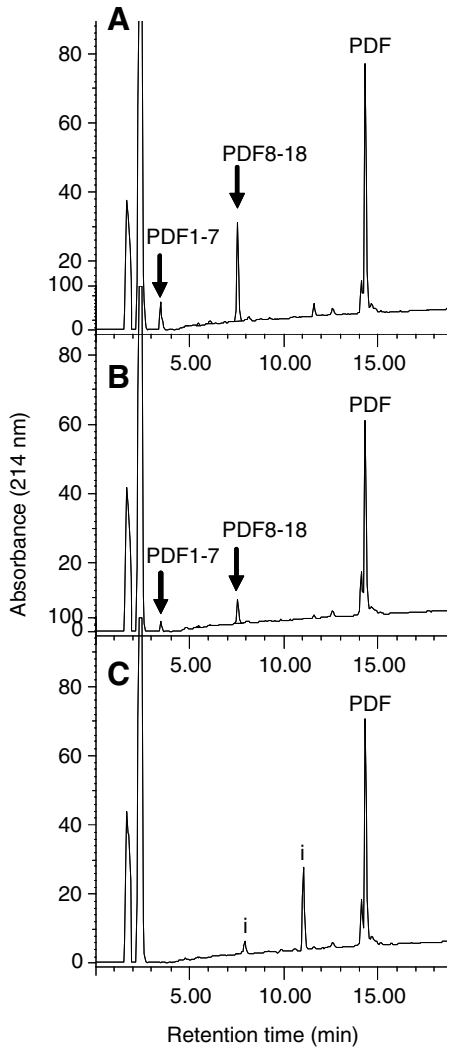

Fig. 2. Hydrolysis of PDF by recombinant human neprilysin and *D. melanogaster* head membranes. Peptide fragments generated after incubation of PDF with either (A) neprilysin (1.25 ng) or (B,C) *D. melanogaster* head membranes (1 μg protein) were separated by HPLC with the UV-monitor set at 214 nm (0.2, full scale absorbance units). The identities of the metabolites PDF1-7 and PDF8-18 were established by mass spectrometry as described in Materials and methods. (C) Pre-incubation of the membranes with 10 μmol l^{-1} phosphoramidon abolished the PDF-degrading activity (I, inhibitor peaks).

Table 1. *Inhibition of PDF hydrolysis by membranes prepared from heads of D. melanogaster*

	% hydrolysis of PDF[a]
No inhibitor	17.5±1.2
Phenanthroline (1 mmol l^{-1})	4.8±1.9
Phosphoramidon (10 μmol l^{-1})	4.1±1.7

[a]PDF hydrolysis was determined by HPLC as described in Materials and methods.
Values are means ± s.e.m., *N*=3.

residues with bulky hydrophobic side chains (Turner, 2004; Oefner et al., 2000). The optimum sequence for a peptide substrate of neprilysin has been determined using model peptides and includes a Leu at the P2′ position, which might explain why cleavage at Ser7–Leu8 is preferred over the Ser9–Leu10 peptide bond (Turner et al., 1985).

A neprilysin-like peptidase is the major PDF-degrading activity of D. melanogaster head membranes

Incubating PDF with membranes derived from *Drosophila* heads at neutral pH resulted in peptide hydrolysis, which was inhibited by the divalent metal chelator 1,10-phenanthroline (73% inhibition) and the neprilysin inhibitor phosphoramidon (77% inhibition) (Table 1). The two major peptide fragments generated by the head membrane peptidases co-chromatographed with PDF1-7 and PDF8-18 (Fig. 2), establishing Ser7–Leu8 as the scissile peptide bond. The formation of these two metabolic products was abolished in the presence of phosphoramidon and thiorphan (Fig. 3; IC_{50} values of 0.15 μmol l^{-1} and 1.2 μmol l^{-1}, respectively), both of which are potent inhibitors of human neprilysin (Turner, 2004). We have considered the possibility that temporal variation in the expression of the PDF-degrading neprilysin(s) might contribute to the circadian functions of PDF, especially since one neprilysin gene (*CG8550*) was shown to cycle in a microarray analysis of circadian gene expression (McDonald and Rosbash, 2001). In four separate experiments, PDF-degrading neprilysin activity was assayed in head membranes prepared from adult *w1118* flies collected at 4 h intervals for 24 h in a 12 h:12 h light:dark cycle. Fluctuations in neprilysin-like activity were apparent; however, the results lacked statistical significance (results not presented). While these results do not demonstrate a role for PDF degradation in regulation of circadian behaviours, we cannot exclude the possibility that localized peptidase activity is under clock control and masked by other peptidases present in whole head extracts.

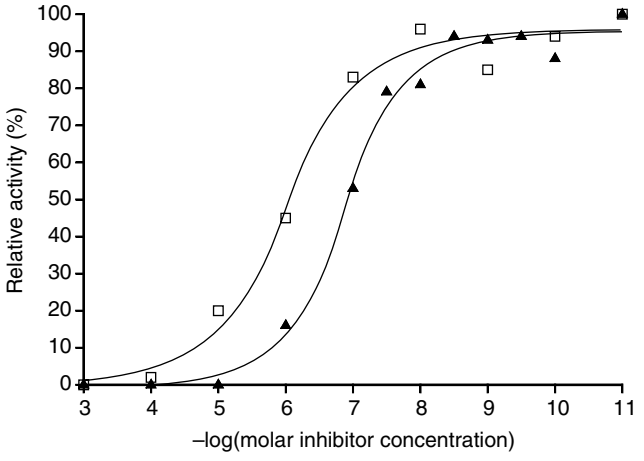

Fig. 3. Inhibition of PDF-degrading activity of *D. melanogaster* head membranes by phosphoramidon and thiorphan. Inhibition curves for phosphoramidon (filled triangles, IC_{50}=0.15 μmol l^{-1}) and thiorphan (open squares, IC_{50}=1.2 μmol l^{-1}) were generated by measuring the formation of PDF8-18 from PDF in the presence of different concentrations of inhibitors, as described in Materials and methods. Data are expressed relative to uninhibited activity and IC_{50} values were calculated using FigP (Biosoft).

PDF1-7 and PDF8-18 do not activate the PDF receptor

Application of PDF1-7 and PDF8-18 on HEK cells co-transfected with the PDF receptor and CRE-luciferase reporter failed to produce significant increases in cAMP (Fig. 4). In contrast, synthetic full-length PDF produced significant increases in cAMP levels, as indirectly measured by luciferase levels. In addition, neither PDF1-7 nor PDF8-18 had a significant effect on the activation of the PDF receptor by the intact PDF ligand. In these experiments, EC_{50} values did not differ significantly (PDF, EC_{50} ~2.4±0.8 μmol l^{-1}; PDF+PDF1-7, EC_{50} ~1.5±0.3 μmol l^{-1}; PDF+PDF8-18, EC_{50} ~1.2±0.4 μmol l^{-1}).

Insect PDF peptides share many structural features with crustacean β-PDH peptides and are active in crustacean pigment

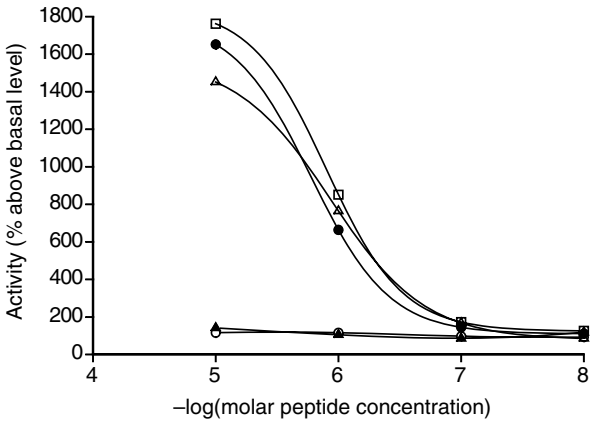

Fig. 4. Dose–response curve for the activation of the PDF receptor of *D. melanogaster* by PDF, PDF1-7 and PDF8-18. The ability of peptides to increase intracellular cAMP levels was determined using HEK293 cells co-transfected with the PDF receptor gene (CG13758) and a firefly luciferase reporter gene construct, as described in Materials and methods. Results are expressed as the mean percentage increase in luciferase activity above basal levels from three replicate wells from three independent transfections (s.e.m.=5–20%). The effects of PDF1-7 and PDF8-18 on the activity of intact PDF were tested by varying the dose of PDF whilst keeping the concentration of each PDF fragment constant at 10^{-5} mol l^{-1}. PDF, solid circle; PDF1-7, open circle; PDF8-18, closed triangle; PDF+PDF1-7, open triangles; PDF+PDF8-18, open square.

dispersion assays (Rao, 2001). All members of the β-PDH/PDF family are octodecapeptides and have a conserved Asn at the N terminus and an amidated C-terminal Ala, and differ in only three or four amino acids (Rao and Riehm, 1989). *Drosophila* PDF differs from the crustacean β-PDH peptides at positions 10 and 14, where Ser and Asn replace Gly and Val, respectively (Park and Hall, 1998). Structure–activity studies suggest that the conserved N and C termini are crucial for full potency of β-PDH/PDF peptides for pigment dispersion in crustacean melanophores, and therefore any cleavage of the peptide should lead to inactivation, at least as regards the pigment dispersion assay (Rao and Riehm, 1989). The amidated C terminus will protect PDF from attack by carboxypeptidases and the presence of Asn at the N terminus might reduce the risk of cleavage of the first peptide bond, since substrates with N-terminal Asn are not noted as good substrates for extracellular aminopeptidases. β-PDH/PDF peptides, however, are vulnerable to cleavage by endopeptidases and since both termini are necessary for full biological activity, at least in crustaceans, such cleavage would almost certainly result in inactivation. In contrast, no structure–activity studies have been performed for PDF in insects, but the recent identification of the *D. melanogaster* PDF receptor and its functional expression in cell lines has now provided the means to conduct such studies (Hyun et al., 2005; Lear et al., 2005; Mertens et al., 2005). Using this assay for receptor stimulation, we have shown that PDF1-7 and PDF8-18 are metabolites with greatly reduced receptor mediated signalling and therefore neprilysin cleavage *in vivo* is likely to terminate the biological actions of PDF.

There are several possible candidates for the PDF-degrading neprilysin from adult *Drosophila* head membranes. The *D. melanogaster* genome encodes 25 neprilysin-like proteins, nine of which lack essential active site amino acids and are therefore unlikely to act as functional peptidases. Analysis of FlyAtlas (Chintapalli et al., 2007) microarray data indicates that at least five of the proteins with intact active sites (NEP1, NEP2, NEP3, NEP4, CG9507) are enriched in either the adult brain or thoracicoabdominal ganglia. Progress in the identification of which neprilysin-like enzyme might be responsible for the extracellular metabolism of neuronal PDF will require the availability of specific antibodies for immunocytochemistry studies to determine which enzyme is located at PDF synapses.

Recent comparisons have been made concerning the phenotypic similarities of PDF and vasoactive intestinal polypeptide (VIP), the major circadian transmitter in mammals (Vosko et al., 2007). Mutations in either of these respective transmitters show similar behavioural phenotypes (Aton et al., 2005; Lin et al., 2004), and these similarities are also evident at the receptor level (Harmar et al., 2002; Mertens et al., 2005). Our results show that PDF is a target of peptidase degradation, and while it remains unclear if this contributes to circadian functions, it is interesting to note that VIP has been found to be degraded by endopeptidases (neprilysin and kallikrein) (Gourlet et al., 1997; Kudo et al., 1998; Wollman et al., 2002) and that in the rat pineal, kallikrein displays a weak circadian rhythm that is antiphasic to VIP (Kudo et al., 1998).

Other peptidases that have been implicated in the degradation and inactivation of insect neuropeptides include members of the angiotensin-converting enzyme (ACE) family, which can cleave dipeptides and dipeptideamides from the C terminus of oligopeptides (Isaac et al., 1998). There are two known active ACE-like enzymes (ANCE and ACER) in *D. melanogaster*, but neither is likely to be involved in the degradation of PDF within the CNS. ANCE is mainly found in the midgut and haemolymph of adults and though ACER is strongly expressed in adult heads, it is not associated with the brain (Chintapalli et al., 2007) (R. E. Isaac, unpublished results). Furthermore, PDF is quite resistant to attack from recombinant ACER (R. E. Isaac, unpublished results)

The molecular identification of the functionally important PDF-degrading peptidase will require the availability of neprilysin-specific antibodies and the matching of the distribution of the peptidase with the PDF receptor. This information will then permit a detailed genetic analysis, localization studies, and behavioural analysis of PDF inactivation by neprilysin peptidases. These studies will undoubtedly offer insight into the regulation of circadian transmitters and their roles within circadian neural circuits.

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