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Double-labelled *in situ* Hybridization Reveals the Lack of Co-localization of mRNAs for the Circadian Neuropeptide PDF and FMRFamide in Brains of the Flies *Musca domestica* and *Drosophila melanogaster*

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Many lines of evidence have suggested that neuropeptides other than pigment-dispersing factor (PDF) are involved in regulating insect circadian rhythms, and FMRFamide-related peptides are additional candidates acting as such neuromodulators. Double-immunolabelling in insect brains with anti-crustacean β-PDH and anti-FMRFamide antibodies had previously suggested that insect PDF and FMRFamide-like peptides may coexist in the same cells. However, it is critical for this kind of comparative investigations to use antibodies of proven specificity, to eliminate the possibility of both reciprocal cross-reactivity and the detection of unknown peptides. In the present study, we achieved the cDNA cloning of an *fmrf* mRNA from the housefly *Musca domestica*, for which co-localization of FMRFamide and PDF peptides was previously suggested. In order to examine the possible co-expression of this gene with the *pdf* gene, we carried out double-labelled *in situ* hybridization for simultaneous detection of both *pdf* and *fmrf* mRNAs in housefly, *Musca* brains. The results clearly indicated that they occur in distinctly different cells. This was also proven for the fruit fly *Drosophila melanogaster* by similar double-labelled *in situ* hybridization. The results thus revealed no reason to evoke the physiological release of FMRFamide and PDF peptides from the same neurons.

Key words: circadian rhythm, double-labelled *in situ* hybridization, FMRFamides, neuropeptides, pigment-dispersing factor (PDF).

Abbreviations: AAP, Abridged Anchor Primer; AP, alkaline phosphatase; AUAP, Abridged Universal Anchor Primer; BCIP, 5-bromo-4-chloro-3-indolylphosphate; DCV, dense core vesicle; DIG, digoxigenin; FaRPs, FMRFamide-related peptides; FITC, fluorescein isothiocyanate; FMRFamide, the one-letter amino acid code denotes H–Phe–Met–Arg–Phe–NH_2; HRP, horseradish peroxidase; NBT, 4-nitro-blue tetrazolium chloride; NTMT, a solution containing 100 mM NaCl, 100 mM Tris–HCl (pH 9.5), 50 mM MgCl2 and 1% Tween 20; PBS, phosphate buffered saline; PBST, PBS containing 0.1% Tween 20; PDF, pigment-dispersing factor; PDH, pigment-dispersing hormone; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription PCR; TBS, Tris-buffered saline; TBST, TBS containing 1% Tween 20.

FMRFamide (H–Phe–Met–Arg–Phe–NH_2) is a neuropeptide originally isolated from the mollusk *Macrocallista nimbosa* and identified by monitoring its cardioexcitatory activity on the clam heart (*1*). Peptides containing C-terminal FMRFamide or a related sequence are members of a large family of structurally related peptides found in both invertebrate and vertebrate species. In insects, these peptides are called FMRFamide-related peptides (FaRPs) and three major families have been reported to date, including FMRFamides, sulfakinins (*2*) and myosuppressins (*3*, *4*). However, little is known about their physiological functions.

Another insect amide peptide, pigment-dispersing factor (PDF), was first found in brains of the grasshopper and its melanophore pigment-dispersing activity monitored in crabs (*5*). Insect PDF is composed of 18 amino acids and involved in the regulation of circadian rhythms as an output neuromodulator (*6–14*). Its involvement in circadian rhythm was shown by the mutant fruit fly *Drosophila melanogaster* designated pdf^{01}. pdf^{01} was found to have a nonsense mutation at the residue 21 of prepro-PDF, converting a Tyr (TA*C*) to a stop codon (TA*A*). This mutant shows abnormal behaviour, the evening activity peak being advanced by approximately 1 h (*11*). The result revealed the substantial importance of *pdf* in circadian system. In order to elucidate the molecular mechanism of PDF peptide in the regulation of circadian rhythms, we have cloned *pdf*

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mRNAs from several insects and clarified the existence of four different types of precursor proteins (*15–19*).

In recent years, there have also been several reports suggesting the involvement of neuropeptides other than PDF in the circadian regulatory system, and FMRFamide peptides have been proposed as candidates for such neuromodulators. Microinjection of FMRFamide peptides into the brains of houseflies, for example, confirmed a strong interrelation between this neuropeptide and circadian rhythms in the visual system (*20*). Double-immunolabelling in insect brains using anti-crustacean β-PDH and anti-FMRFamide antibody had suggested moreover that insect PDF and FMRFamide-like peptides may co-exist in the same cells in several different insect species (*20–23*). However, immunocytochemical investigations crucially require the use of highly specific antibodies against each peptide in order to avoid possible cross-reactivity to unknown peptides.

As a check, multiple-labelling *in situ* hybridization techniques can provide sensitive cytochemical information about more than two different mRNA genes in the same preparation. This method requires the precise nucleotide sequence of a target mRNA in the preparation of cRNA probes. The recent cDNA cloning and *in situ* hybridization of *pdf* mRNA of the house fly *Musca domestica* (*17*), provided an excellent opportunity to examine the co-localization of *pdf* with *fmrf* mRNA in the *Musca* brain. *Musca* has a brain size bigger than that of the fruit fly *Drosophila* and this is definitely advantageous to identify the mRNA-expressing cells as a prior experiment to the analysis for *Drosophila* brain. Thus, in the present study we first carried out molecular cloning of *Musca fmrf* mRNA.

For insect FMRFamide-related peptides, available cloning data have hitherto been limited to two different fruit fly species, *D. melanogaster* and *D. virilis*. The *D. melanogaster fmrf* gene was identified by screening the genomic library using *fmrf* cDNA sequence data from the marine mollusk *Aplysia californica* (*24*) and eventually found to encode 13 copies of 8 different FaRPs, five copies of which are exactly identical (*25*). The *fmrf* gene of *D. virilis* was also cloned by screening the genomic library using the *D. melanogaster* genome fragment lying 5′ upstream of the *fmrf* mRNA start site (*26*). This *fmrf* gene encodes 10 different FaRPs, six of which are different from *D. melanogaster* FaRPs. Although such a low sequence similarity makes the search for *fmrf* genes in insects very difficult, based on this sequence information we planned to clone the housefly *fmrf* gene. Here we describe the results of cDNA cloning of the *Musca fmrf* gene and double-labelled *in situ* hybridization of both *fmrf* and *pdf* mRNAs.

MATERIALS AND METHODS

Animals—The housefly *Musca domestica* was purchased from Sumika Technoservice Co. (Takarazuka). All flies were maintained at 25°C under a day:night cycle of L12:D12. To collect brain samples for cDNA cloning, the flies were dissected under a microscope and their brains were immediately frozen in liquid nitrogen. Dissected thoracic ganglia of males and females were stored at −80°C until use. For *in situ* hybridization, 5-day-old female houseflies were used.

3′ RACE for Identification of 3′ End Coding Musca fmrf cDNA—The cDNA cloning of the *Musca fmrf* gene was carried out by the 3′ RACE method (*27*). mRNAs were extracted from the thoracic ganglia dissected from about 30 flies using a QuickPrep® *Micro* mRNA Purification Kit (Amersham Biosciences, Piscataway, NJ, USA) according to the Manufacturer's instructions. The mRNAs (300 ng) were reverse-transcribed by Super Script™ II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) using $d(T)_{17}$-adapter primer (5′-GGCCACGCGTCGACTAGTAC-T_{17}-3′) at 42°C, as previously described (*16*). The resulting cDNAs were subjected to the polymerase chain reaction (PCR) using adaptor primer (5′-GGCCACGCGTCGACTAGTAC-3′) and degenerated primers PKQDFMRFG-F (5′-CCIHVI CARGAYTTYATGMGITT-3′), QDFMRFGR-F (5′-CARG AYTTYATGMGITTYGGIMG-3′) or DNFMRFGR-F (5′-GAYAAYTTYATGMGITTYGGIMG-3′). In these primers, the letters H, V, M, R and Y denote the nucleotides (not G), (not T), (A or C), (A or G) and (C or T), respectively. The primer was designed based on amino acid sequence homology between the FaRP peptide sequences for *D. melanogaster* and *Calliphora vomitoria* (*28*). PCR was performed using AccuPrime™ *Taq* DNA polymerase (Invitrogen) with a slight modification for touch-down PCR condition (*29*): *i.e*. the reaction condition used, 3 min at 94°C followed by 3 cycles of 94°C for 30 s, 68°C for 30 s, 72°C for 60 s, 3 cycles of 94°C for 30 s, 65°C for 30 s, 72°C for 60 s, 3 cycles of 94°C for 30 s, 63°C for 30 s, 72°C for 60 s, 3 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 60 s, 3 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 60 s, 25 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 60 s and a final extension for 7 min at 72°C. The PCR products were subcloned into pBluescript II SK+ and the sequence was analysed by a Thermo Sequenase Cy5.5 dye terminator cycle sequencing kit (Amersham Biosciences).

On the basis of the *Musca fmrf* sequence so obtained, gene-specific primers were newly designed and 3′ RACE was performed for a second time. The primers used were MuscaFMRF-F1 (5′-TGGCCGTAGTCCAGGAAGCCAA-3′ corresponding to the nucleotide sequence 1008–1029 in Fig. 1) and MuscaFMRF-F2 (5′-GCATCGGGTGGACA AGACTTCAT-3′ corresponding to the nucleotide sequence 1159–1181 in Fig. 1). The sequence analysed was confirmed by sequencing several other clones simultaneously.

5′ RACE for Identification of 5′ End Encoding Musca fmrf cDNA—To amplify the 5′ end of the *M. domestica fmrf* (denoted hereafter as *Musca fmrf*) cDNA, 5′ RACE (*26*) using the 5′ RACE System for Rapid amplification of cDNA ends Version 2.0 (Invitrogen) was performed according to the Manufacturer's protocol with some modifications. First-strand cDNA was synthesized from an mRNA by Super Script™ II reverse transcriptase (Invitrogen) with a *Musca fmrf*-specific antisense primer MuscaFMRF-R3 (5′-AAGCTCATGTTTATCGAAT-3′, corresponding to the nucleotide sequence 1485–1503 in Fig. 1) at 42°C. The product was digested by RNase H/T_1 to remove the original mRNA template.

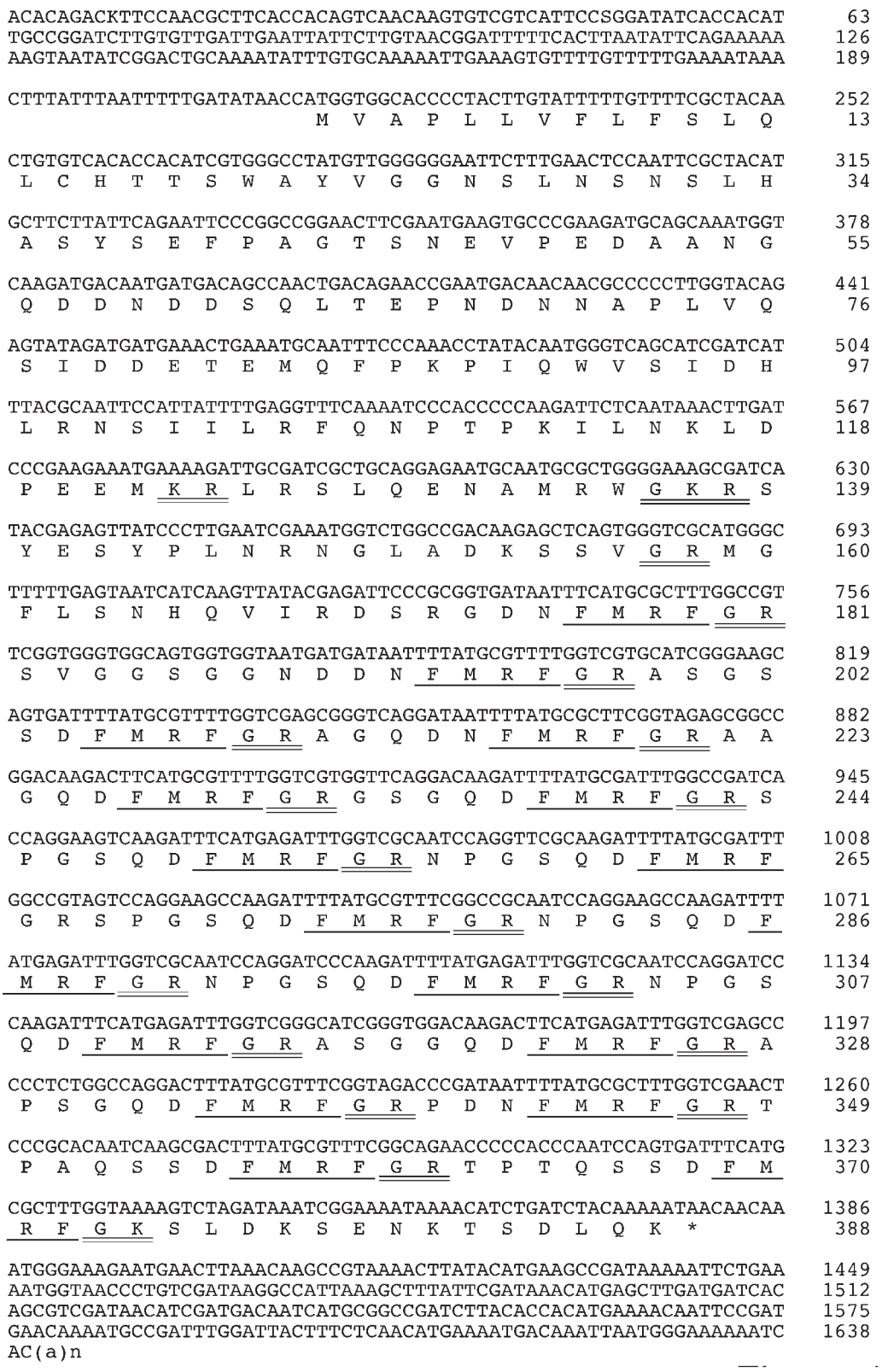

Fig. 1. **Nucleotide and deduced amino acid sequences of *Musca* FMRFamide.** A full-length cDNA clone comprises 1640 bp encoding a precursor protein of 388 amino acid residues. The FMRF structures, Phe–Met–Arg–Phe, are underlined, and the dibasic KR sites and monobasic sites GR and GK are double-underlined.

A homopolymeric tail poly(C) was elongated by using terminal deoxynucleotidyl transferase and a substrate dCTP. The resulting tailed cDNA was amplified by the Abridged Anchor Primer AAP (5′-GGCCACGCGTCG ACTAGTACGGGIIGGGIIGGGIIG-3′ provided by the kit, Life Tech.) and a *Musca fmrf* gene-specific antisense primer MuscaFMRF-R2 (5′-GGCCTTATCGACAGGGT TACCATTT-3′, corresponding to the nucleotide sequence 1449–1473 in Fig. 1) under the conditions essentially as for 3′ RACE. The PCR product was further amplified with Abridged Universal Anchor Primer (AUAP) (5′-GGC CACGCGTCGACTAGTAC-3′, Invitrogen) and *Musca fmrf* gene-specific antisense nested primer MuscaFM RF-R1 (5′-CTTCATGTATAAGTTTTACGGCTTGTT-3′ corresponding to the nucleotide sequence 1406–1432 in Fig. 1). The respective PCR and sequence analyses were carried out as described earlier, and the sequence analysed was confirmed by sequencing several different clones simultaneously.

In order to obtain the authentic full-length 5′ end, further 5′ RACE was performed by the procedure described earlier. One modification was to increase the temperature in a reverse transcription reaction by using newly designed primers. First-strand cDNA was prepared from mRNA with MuscaFMRF-R2 at 55°C, about 10°C higher than that in the previous run. The PCR amplification reaction was performed with MuscaFMRF-R1 and AAP primers, and nested PCR was carried out with a newly designed *Musca fmrf* gene-specific primer MuscaFMRF-R0 (5′-AGCCCATGCGACCCACTGAGCTC TT-3′ corresponding to the nucleotide sequence 670–694 in Fig. 1) and AUAP. All other reactions were executed as mentioned earlier.

In situ Hybridization of fmrf mRNA Gene—Fixation of Musca Brains—Brains of 5-day-old houseflies *Musca domestica* were dissected under a binocular microscope and fixed in freshly made 4% formaldehyde, as paraformaldehyde, in 0.1 M phosphate buffer (pH 7.4) for 12 h at 4°C. Tissues were washed at 4°C with 0.01 M PBS containing 0.1% Tween 20 (denoted as PBST), dehydrated with a series of MeOH solutions (25, 50, 75% and then 2 × 100%) in PBST, and stored at −20°C until use.

Preparation of cRNA Probes for Musca fmrf and pdf mRNA Genes—To clarify the location of the cells expressing *fmrf* mRNA, a digoxigenin (DIG)-labelled *fmrf* cRNA probe was prepared. Using T7 RNA polymerase, an antisense *fmrf* cRNA probe labelled by digoxigenin (DIG)-UTP was produced *in vitro* from plasmid linearized with *Sal*I. The plasmid used involved the PCR product corresponding to *fmrf* cDNA of position 1159–1592 in the vector pBluescript II SK+. The reaction mixture of plasmid (1 μg), 5× buffer (4 μl; 0.2 M Tris–HCl (pH 8.0), 40 mM $MgCl_2$, 10 mM spermidine-$(HCl)_3$, 125 mM NaCl), 10× DIG RNA labelling mix (2 μl; 10 mM ATP, 10 mM CTP, 10 mM GTP, 6.5 mM UTP, 3.5 mM DIG-11-UTP (pH 7.5; Roche Diagnostics, Mannheim, Germany), 5 mM DTT (2 μl), an RNase inhibitor RNasin® (1 μl; Promega, Madison, WI, USA) and T7 RNA polymerase for antisense primer (2 μl, 100 U) was incubated at 37°C for 2 h. The reaction was terminated by adding 0.5 M EDTA (2 μl, pH 8.0). In order to precipitate DIG-labelled cRNA product, 4 M LiCl (2 μl) and ethanol (75 μl) was added and the solution was centrifuged at 17,000 *g* for 20 min at 4°C. The residual pellet was washed with 70% ethanol and dried in the air. The pellet was eventually dissolved in 50% formamide (50 μl). The quality of the transcript was analysed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide.

For double-labelled *in situ* hybridization, DIG-labelled *fmrf* cRNA prepared above was utilized as one of the probes. In addition, as a counterprobe, a fluorescein isothiocyanate (FITC)-labelled *pdf* cRNA probe was prepared. Using T7 RNA polymerase, antisense *pdf* cRNA probe labelled with FITC-UTP was prepared *in vitro* from the plasmid linearized with *Bam*HI, corresponding to the nucleotide positions 39–501 of *Musca pdf* (GenBank accession No. AB095922). *In vitro* transcription was performed using an FITC RNA labelling mix (Roche) instead of the DIG RNA labelling mix.

Whole-mount in situ Hybridization—Whole-mount *in situ* hybridization was performed essentially as described by Wilkinson (*30*) with several significant modifications. All the following procedures were performed at 25°C unless otherwise noted. Dehydrated tissues were rehydrated through a reverse series of MeOH–PBST solutions (75, 50, 25% MeOH, and 2× PBST). These re-hydrated tissues were permeabilized with 10 μg/ml proteinase K (Nacalai Tesque, Kyoto) at 37°C for 15 min, washed with aqueous glycine (2 mg/ml) for 5 min, and then washed twice with PBST. They were fixed in 0.2% glutaraldehyde/4% paraformaldehyde in PBST for 20 min, washed twice with PBST and incubated in PBST at 70°C for 50 min. After cooling on ice, tissues were treated with 6% H_2O_2 for 1 h, washed three times with PBS and incubated with pre-hybridization buffer (50% formamide, 5× SSC (pH 4.5), 1% SDS, 50 μg/ml yeast tRNA and 50 μg/ml heparin) at 70°C for 1 h. The pre-hybridization buffer was then replaced with hybridization buffer, *i.e.* a solution of pre-hybridization buffer containing a DIG-labelled cRNA probe, and the solution was incubated at 70°C for 16 h.

After hybridization, tissues were washed three times with solution I [50% formamide, 5× SSC (pH 4.5) and 1% SDS] at 70°C for 30 min, and once more with a 1:1 mixture of solutions I and II (solution II: solution I with no formamide) at 70°C for 10 min. The tissues were washed further three times with solution II for 5 min, and then incubated with solution II for 20 min. Washings were repeated twice with solution III [50% formamide, 2× SSC (pH 4.5) and 1% SDS] for 5 min at 70°C, three times more with solution III, but for 30 min each, and then with a TBST solution in which Tris-buffered saline (TBS; 150 mM NaCl, and 100 mM Tris–HCl, pH 7.5) contains 1% Tween 20. After blocking at 25°C for 1 h, in order to visualize mRNA-bound probe by immunocytochemical detection, tissues were treated with Fab fragments of sheep anti-DIG antibody directly conjugated to alkaline phosphatase (AP). This incubation was continued for 16 h at 4°C. Tissues were then washed successively four times with TBST for 5 min each and seven times for 1 h each. After washing twice with NTMT [100 mM NaCl, 100 mM Tris–HCl (pH 9.5), 50 mM $MgCl_2$ and 1% Tween 20] for 5 min, the antibody detection

reaction was performed by incubating the tissues in the substrate solution [NTMT with 0.33 mg/ml 4-nitro-blue tetrazolium chloride (NBT) and 0.17 mg/ml 5-bromo-4-chloro-3-indolylphosphate, BCIP] for 30 min in shaded light, and finally washed with TBST. Finally, whole tissues were mounted in glycerol–gelatin (Sigma, St Louis, MO, USA) and observed by light microscopy with an Olympus BX50.

Double-labelled in situ Hybridization—Double-labelled *in situ* hybridization was performed by means of the procedure used for whole-mount *in situ* hybridization with some modifications. Housefly brains were incubated in the hybridization solution containing both DIG-labelled *fmrf* cRNA probe and FITC-labelled *pdf* cRNA probe for 16 h at 70°C. After this hybridization reaction, tissues were washed as described for the whole-mount *in situ* hybridization and brains were subjected to the blocking reaction. After blocking at 25°C for 1 h, tissues were treated with anti-DIG-peroxidase (POD) Fab fragments (Roche) for 16 h at 4°C. Tissues were washed and DIG-labelled *fmrf* cRNA signals were amplified by TSA™ plus dinitrophenol (DNP) technology. Briefly, tissues were treated with DNP working solution for 10 min and washed. After blocking, tissues were incubated with anti-DNP-HRP Fab fragments and signals were visualized by the ImmunoPure® Metal Enhanced DAB (dimethylaminoazobenzene) Substrate kit (Pierce), which utilizes cobalt chloride and nickel chloride to produce a dark-brown precipitate in the presence of horseradish peroxidase (HRP). After washing with TBST, housefly brains were incubated with anti-FITC-AP Fab fragments for 16 h and then with the substrate solution (NTMT containing BCIP/NBT) for 30 min in shaded light. Eventually, the whole tissues were washed with TBST and mounted in glycerol–gelatin (Sigma) to observe by light microscopy as before.

Double-labelled in situ Hybridization of Drosophila fmrf and pdf mRNA Genes—Double-labelled *in situ* hybridization of *Drosophila* (Canton S) *fmrf* and *pdf* mRNA genes was carried out using a DIG-labelled *fmrf* cRNA probe and an FITC-labelled *pdf* cRNA probe, respectively. Antisense *pdf* cRNA probe labelled with FITC-UTP was prepared *in vitro* from the phagemid linearized with *Eco*RI. This ca. 600 bp probe corresponds to the whole *Drosophila pdf* mRNA (GenBank accession No. NM079793). Antisense *fmrf* cRNA probe labelled by DIG-UTP was prepared in a similar way from plasmid involving the PCR product corresponding to *Drosophila fmrf* cDNA (GenBank accession No. NM139501) of position 656–1473. Fixation followed by double-labelled *in situ* hybridization was performed under the same conditions for *Musca*.

RESULTS

Musca domestica fmrf cDNA—Degenerate primers for cDNA cloning were designed with reference to the sequence homology between FMRFamide peptides of *Drosophila* and *Calliphora vomitoria* (*28*, *31*, *32*). For the 3′ RACE method, three primers, PKQDFMRFG-F, QDFMRFGR-F and DNFMRFGR-F, were used to amplify the *Musca fmrf* gene. Several distinct gel bands were detected in each PCR reaction using these primers, and the nucleotide sequences of all the resulting products were analysed. In short, only when DNFMRFGR-F was used the fragment of *Musca fmrf* gene was obtained and this product was found to contain five copies of FMRF (corresponding to 274–325 in the final amino acid sequence). 3′ RACE and 5′ RACE experiments were performed on the basis of sequence information of this fragment.

A full-length cDNA clone was finally obtained, and the entire oligonucleotide structure was clarified. It comprises 1640 bp encoding a precursor protein of 388 amino acid residues (Fig. 1). Since the selected ATG codon and its adjacent nucleotide sequences fulfilled Kozak's consensus motif (*33*), the initiator codon ATG was assigned to position 214–216 as shown in Fig. 1. No additional ATG codon was found in the upstream 5′ UTR region. The program Signal P (http://www.cbs.dtu.dk/services/SignalP/) for predicting a signal peptide cleavage site showed that 21 amino acids from a selected Met should be a signal peptide.

Amino Acid Sequence Analysis of FMRFamide Peptides—The amino acid sequence converted from the oligonucleotide sequence of the *Musca fmrf* cDNA gene revealed that the gene encodes 17 FaRPs in total. At the most C-terminal side, the precursor contains a C-terminal free 14-mer peptide. At the N-terminal side, there is a 101-mer peptide (or protein) truncated by the 22-mer N-terminal signal peptide and the KR cleavage site. Consecutively, there follow an 11-mer peptide amide LRSLQENAMRW-NH_2(GKR) and then an 18-mer peptide amide SYESYOLNRNGL ADKSSV-NH_2(GR). All 17 FaRPs are present between this 18-mer peptide amide and C-terminal 14-mer peptide. They are encoded in tandem, being truncated by the amidation signal of GR at 16 sites and GK at one site (Fig. 2). As shown in Fig. 2, the size of the *Musca* FaRPs is relatively small, with amino acid residues 7–21.

Among total of 17 copies of FaRPs, there are four copies of NPGSQDFMRF-NH_2(GR) and two copies of SPGSQDFMRF-NH_2(GR) (Fig. 2). Interestingly, these peptides have exactly the same sequence of PGSQ DFMRF-NH_2(GR), and only the N-terminal amino acids N(=Asn) and S(=Ser) are different from each other. When we designate these peptides NPGSQDFMRF-NH_2(GR) and SPGSQDFMRF-NH_2(GR) as (*N*) and (*S*), respectively, they were found to be encoded successively in tandem as in the order of (*S*)–(*N*)–(*S*)–(*N*)–(*N*)–(*N*). Other 11 of the FaRPs have an N-terminal peptide sequence of MGFLSNHQVIRDSRGDN, SVGGSGGN DDN, ASGSSD, AGQDN, AAGQD, GSGQD, ASGGQD, APSGQD, PDN, TPAQSSD or TPTQSSD, as well as the C-terminal FMRF-NH_2(GR). In *D. melanogaster*, 8 different kinds of FaRPs, which total 13 copies, are encoded in a single gene (Fig. 2). Among them, only PDNFMRF-NH_2(GR) was found to be shared between the *Drosophila* and *Musca* FaRPs.

fmrf-Expressing Cells in Musca Brains—Whole-mount *in situ* hybridization was performed to identify the cells expressing *fmrf* mRNA. A DIG-labelled cRNA probe

Fig. 2. **Various FMRFamide-related peptides present in the precursor proteins of the housefly *Musca domestica* (A) and the fruit fly *Drosophila melanogaster* (B).** The signal peptides shown by dotted underline were predicted by program Signal P (http://www.cbs.dtu.dk/services/SignalP/). Dibasic amino acid cleavage sites KR are double-underlined. All 'FMRF' sequences are boxed. A solo 'GK' sequence present in *Musca* FMRF amide is underlined. Asterisks indicate a stop codon there.

was prepared to hybridize the *fmrf* mRNA gene at positions 1159–1592, which corresponds to the 3′ portion (1159–1380) of the protein-coding region and 3′ UTR (1381–1592). As shown in Fig. 3A, approximately four cell groups were identified for the *Musca* brain by this whole-mount *in situ* hybridization procedure. One large group, with two cell clusters each containing two large cells, was observed in the lateral neurons in each optic lobe (Fig. 3B), and two slightly bigger cells were observed on both sides of the tritocerebrum (Fig. 3C). A cell group of about 14 fairly small cells was observed in the dorsal area (Fig. 3D), while another group of 13 cells was in the subesophageal ganglion (Fig. 3E). The arrangement of these clusters is summarized in Fig. 4. In addition to the lateral neurons, most *fmrf* mRNAs are expressed in the central brain. No signals were detected when used sense *fmrf* mRNA probe as negative control (data not shown).

It is significant that some *Musca fmrf* mRNA-expressing cells appeared to locate in the same portion of the brain as in the *Musca* brain, containing *Musca pdf* mRNA-expressing cells. As previously reported (*13*), these are the lateral neurons of the optic lobe. There are two *pdf* expressing cell clusters in each optic lobe, one comprising four large cells and the other four small cells, as seen with double-labelled *in situ* hybridization, below. The results from *in situ* hybridization of *fmrf* mRNA show that the two *fmrf*-expressing cell clusters are located among the lateral neurons and that their cell sizes are similar to those of the large *pdf*-expressing cells.

Double-labelled in situ Hybridization of fmrf- and pdf-expressing Cells—In situ hybridization experiments carried out individually to detect *fmrf* and *pdf* mRNAs, raised the possibility that, among the *fmrf*-expressing cells, two pairs of lateral neurons could also express *pdf* mRNA (Fig. 4). To confirm whether or not *fmrf* and *pdf* mRNAs are co-expressed in the exact same cells, double-labelled *in situ* hybridization was performed using the DIG-labelled cRNA probe to detect *fmrf* mRNA and FITC-labelled cRNA probe for *pdf* mRNA (Fig. 5A). Both signals were found in the same region of fly's brain containing the lateral neurons, the anterior cortex of the medulla. However, as shown in Fig. 5B,

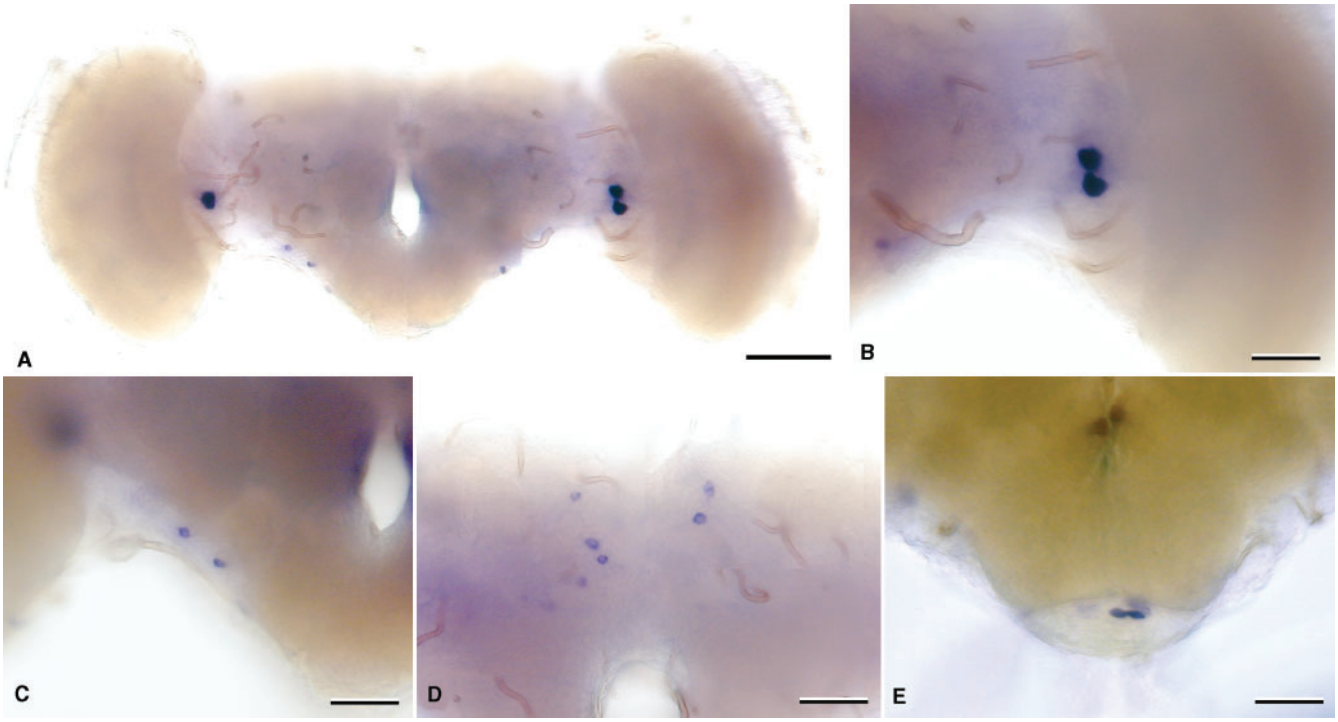

Fig. 3. **The whole-mount *in situ* hybridization for detection of *fmrf* mRNA in the brain of the housefly *Musca domestica*.** (A) A profile of whole brain with optic lobes (scale bar, 100 μm); and (B), (C), (D) and (E) expanded segmental views (scale bar, 50 μm). Observed cells are as follows: (B) two distinct cells in the lateral neurons in each optic lobe; (C) a single cell in the tritocerebrum; (D) approximately 14 cells in the dorsal area and (E) approximately 13 cells in the subesophageal ganglion.

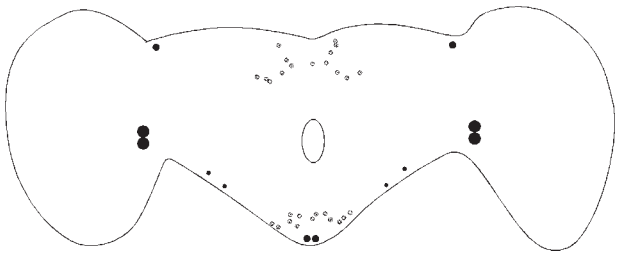

Fig. 4. **Schematic perspective drawing of the *fmrf* mRNA-expressing cells in the brain of the housefly *Musca domestica* detected by whole-mount *in situ* hybridization.** There are two pairs of four large cells among the lateral neurons, and two slightly bigger cells in the tritocerebrum. In addition, approximately 14 small cells are in the dorsal portion.

both brown and dark-blue signals never merged with each other. Two *fmrf*-expressing cells and all of *pdf*-expressing cells, four large and four small cells, were identified separately and individually in the lateral neurons. The *fmrf*-expressing cells revealed by brown signals were found more ventrally than the large *pdf*-expressing cells shown by dark-blue signals, and more lateral and dorsally than the small *pdf*-expressing cells. Given that the two signals nowhere merged, the *fmrf*-expressing cells are clearly different from the *pdf*-expressing cells.

The brains of *D. melanogaster* also exhibited non-merging signals of the *fmrf* mRNA hybridized by DIG-labelled cRNA probe and of the *pdf* mRNA hybridized by FITC-labelled cRNA probe. As shown in Fig. 6, with the FITC-labelled cRNA probe, *Drosophila pdf* mRNAs were observed in both large and small lateral neurons (*l*LNs and *s*LNs). When hybridized with DIG-labelled cRNA probe, *fmrf* mRNA-expressing cells were also found in the same region of the brain as the *pdf* mRNA-expressing *l*LNs and *s*LNs. However, again, the respective signals never merged, indicating that *fmrf*-expressing cells are clearly different from *pdf*-expressing cells.

DISCUSSION

Multiplicity of FMRFamide Peptides in the Amino Acid Sequence—Since the discovery of tetrapeptide FMRFamide, numerous members of the FaRP peptide family have been identified throughout the Metazoa. The *fmrf* mRNA was first clarified from an abdominal ganglion cDNA library of the marine mollusk *Aplysia californica* by a differential screening technique (*25*). Surprisingly, in addition to the FLRF tetrapeptide amide (=FLRFGR) and GYLRF pentapeptide amide (GYLRFGR), it encoded 28 FMRFs, the tetrapeptide amide FMRFGR, in tandem in a single gene. Multiple FaRPs are also encoded in other invertebrates, for instance, in the nematode *Caenorhabditis elegans*. *C. elegans* has no less than 20 FaRP precursor genes which encode a total of 56 FaRPs (*34*, *35*). No physiological significance of such diversity and multiplicity has yet been clarified.

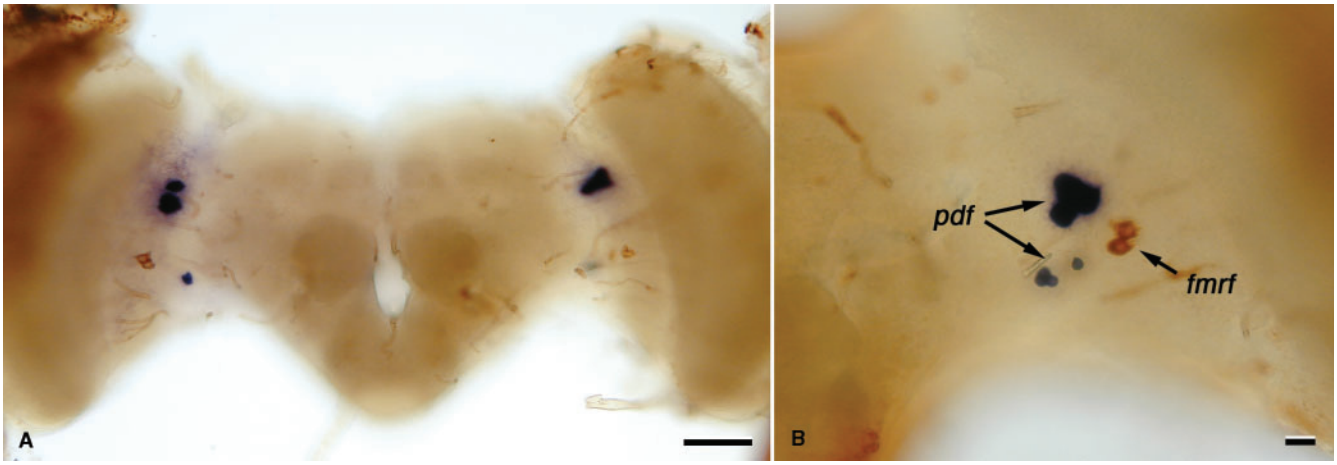

Fig. 5. **Double-labelled *in situ* hybridization of *fmrf* and *pdf* mRNAs in the brain of the housefly *Musca domestica*.** Brown signal indicates cells expressing *fmrf* mRNA, while dark-blue signal indicates those expressing the *pdf* mRNA. (A) Entire brain treated with double-labelled *in situ* hybridization (scale bar, 100 μm); and (B) expanded sectional view (scale bar, 20 μm).

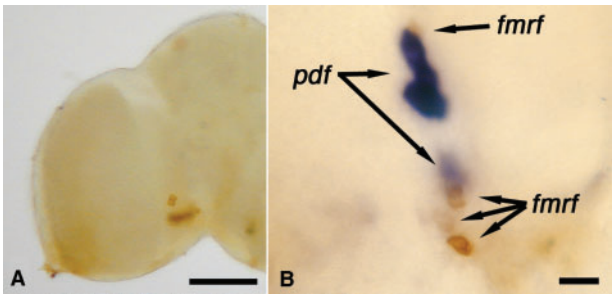

Fig. 6. **Double-labelled *in situ* hybridization of *fmrf* and *pdf* mRNAs in the brain of the fruit fly *Drosophila melanogaster*.** Brown signal indicates cells expressing *fmrf* mRNA, while dark-blue signal indicates those expressing the *pdf* mRNA. (A) Entire brain treated with double-labelled *in situ* hybridization (scale bar, 100 μm); and (B) expanded sectional view (scale bar, 20 μm).

In the EMBL/Genbank database, only two nucleotide sequences for FMRFamide peptides have been reported, the mRNAs of the fruit flies *D. melanogaster* and *D. virilis*. Indeed, such very limited information regarding the oligonucleotide sequences provided severe obstacles to the cDNA cloning of the housefly *fmrf* mRNAs, and required very sophisticated experimental strategies and primer design for the eventual successful cloning. For sufficient gene amplification, PCR should be carried out under the condition that there is an adequate number of target clones in the cDNAs that are reverse-transcribed. It was therefore a concern that the concentration of *fmrf* mRNA in the whole brain. In order to overcome this intrinsic problem, mRNAs were finally extracted from the thoracic ganglia. The fact that thirteen different FaRP peptides were actually isolated from the thoracic ganglia of the blowfly *Calliphora vomitoria* (*28*) prompted us to excise this tissue rather than the brain for the collection of concentrated *fmrf* mRNA. The difficulty in cDNA cloning further became clear when the amino acid sequence of the precursor protein of FMRFamide peptides was explored. Its sequence, particularly the sequences of FMRFamide peptides, was found to differ considerably from those of *D. melanogaster* (Fig. 7). The differences were remarkable; with only one FMRFamide peptide conserved between the two fly species, even though both are members of closely related clades of Diptera. It is evident that cDNA cloning of such a divergent gene is difficult.

When the amino acid sequences of precursor proteins translated from *Drosophila* and *Musca fmrf* mRNAs were compared (Fig. 2), these FaRP genes were found to show various disparities. First of all, the total numbers of copies of FMRFamide-containing peptides encoded in a single gene are different: 10 copies for *Drosophila* and 17 for *Musca*. Among the 17 *Musca* FMRFamide peptides, the peptides encoded at the most N-terminal side are 21- and 16-mer FMRFamides. All other FMRFamide peptides are rather short with 7–11 amino acid residues. Most FMRFamide peptides are truncated by GR at the both the N- and C-terminal sides, whereas the GR and GK sites truncate the most C-terminal TPTQSSDFMRFamide. Thus, there are 16 FMRFGR sequences in total and 1 FMRFGK in the same gene. Although the monobasic sites GR and GK are not always enzymatically cleavable (*36*), these are assumed to be the truncate sites for exclusive production of FMRFamide peptides. *Drosophila* has one longer-type (16-mer) and nine shorter-type (7–9-mer) FMRFamide peptides as shown in Fig. 7.

As to the FMRFamide peptides of the shorter type, there are remarkable differences in their primary structures. *Musca* FMRFamide encodes eight different 10-mer peptides, which contain a hexapeptide amide of QDFMRFamide in their C-terminus. The *Drosophila* FMRFamide precursor consists of six different peptides terminating with C-terminal QDFMRFamide, but in 9-mer peptides. These suggest an important role for the physiological functions of the QDFMRFamide moiety, presumably as an address code to specify and bind to specific receptors.

fmrf-Expressing Cells do not Express pdf—PDF peptide in *D. melanogaster* is present in several cell clusters

```
DmelanogasterFMRF  MGIALMFLLALYQMQSAIHSEIIDTPN-YAGNSLQDADSQVSPS----QDNDLVDALLGN 55
DvililusFMRF       MGIALMFLLALYQMQSAIHSEIIETPSSYNDNSLLEAAAEEPNSRATASESDLLDGLMST 60
MuscaFMRF          MVAPLLVFLFSLQLCHTTSWAYVGGNS-LNSNSLHASYSEFPAGTSNEVPEDAANGQDDN 59
                   *  .*:.:*   *:  :     :   .   .***  : :: . .      .*  :.  ..

DmelanogasterFMRF  DQ-----------------TERAELEFRHPISVIGIDYSKNAVVLHFQKHGRKPRYKYDP 98
DvililusFMRF       DNP--------------NPEQQTELEFRYPISAIGIGYAKNSVVLRFQKHARKQNFKYDP 106
MuscaFMRF          DDSQLTEPNDNNAPLVQSIDDETEMQFPKPIQWVSIDHLRNSIILRFQNPTPKILNKLDP 119
                   *:                  :.:*::*  **. :.*.: :*:::*:**:   *   * **

DmelanogasterFMRF  ELEAKRRSVQDNFMHFGKRQAEQLPPEGSYAGSDELEGMAKRAAMDRYGRDPKQD-FMRF 157
DvililusFMRF       DYEMKRKSLQDNFMHFGKRQAEQLP-QATGPG---YYECIKRSAMDRYGRDPKQD-FMRF 161
MuscaFMRF          EEMKRLRSLQENAMRWGKRSYESYPLNRNGLADKSSVGRMGFLSNHQVIRDSRGDNFMRF 179
                   :   : :*:*:* *::***. *. * : .  .          : .:  **.: * ****

DmelanogasterFMRF  GRDP------KQDFMRFGRDPKQDFMRFGRDPKQDFMRFGRDPKQDFMRFGRTPAADFMR 211
DvililusFMRF       GRAP------PSDFMRFGRAP-----------------------------------SDFMR 181
MuscaFMRF          GRSVGGSGGNDDNFMRFGRASGSS----------DFMRFGRAGQDNFMRFGRAAGQDFMR 229
                   **          .:****** .                                    ****

DmelanogasterFMRF  FGRTPAEDFMRFGRS---DNFMRFGRSP--HEDVR---SP-KQDFMRFGR---PDNFMRF 259
DvililusFMRF       FGRDPSQDFMRFGRS---DNFMRFGRNLNFHEELR---SP-KQDFMRFGR---PDNFMRF 231
MuscaFMRF          FGRGSGQDFMRFGRSPGSQDFMRFGRNPGSQDFMRFGRSPGSQDFMRFGRNPGSQDFMRF 289
                   *** ..:********   ::******.   :: :*   ** .********   .::****

DmelanogasterFMRF  GRS-RPQDFVRSGK--MDSNFIRFGKS------LKPAAPESKPVKSNQGN--PGER---S 305
DvililusFMRF       GRS-APTEFERNGK--MDSNFMRFGKRSGVMAKLTKSQLQQNKLTTADGKQQPAEEG--N 286
MuscaFMRF          GRNPGSQDFMRFGRNPGSQDFMRFGRASGGQDFMRFGRAPSGQDFMRFGRPDNFMRFGRT 349
                   **.  . :* * *:   ..:*:***:       :  .   .       *.     .   .

DmelanogasterFMRF  PVDKAMTELFKKQELQDQ-------------QVKNGAQATTTQDGSVEQDQFFGQ 347
DvililusFMRF       PTDKAISMLFNKHQQQQQQQQQGQRLQQEDRQQMKSSAEQNNLEEASVEQ--FYEP 339
MuscaFMRF          PAQSSDFMRFGRTPTQSS----------DFMRFGKSLDKSENKTSDLQK------ 388
                   *.:.:    * :   *..             :. .. : .  : ..:::
```

Fig. 7. **The sequence alignment of fly FMRFamide precursor proteins.** '∗': Residues in that column are identical in all sequences in the alignment. ':': Conserved substitutions are observed. '.': Semi-conserved substitutions are observed.

of clock cells that express the clock gene *period*. There is clear evidence that the *pdf*-mutated flies show abnormal circadian rhythm behaviour (*11*), although these mutant flies do not show arrhythmic, completely random activity. Residual rhythmicity in *pdf* mutants and the residual *period*-expressing cells which lack *pdf* peptide likely reflects the activity of other neurotransmitters. It has also been reported that certain mosaic flies for the neuropeptide amidating enzyme were less rhythmic than *pdf* mutants (*37*). This suggests that multiple amidated neuropeptides other than PDF function in daily locomotor rhythms.

Together with these suggestions, several other investigations strongly suggested that (*1*) FMRFamide peptides are a neuropeptide involved in circadian rhythm (*20*); and (*2*) FMRFamide peptides are co-expressed or co-localized with PDF peptide in the same neurons (*20–23*). However, the latter possibility was clearly excluded in the present study by means of double-labelled *in situ* hybridization, at least for the two flies *M. domestica* and *D. melanogaster*. In *Musca* brains, a cell cluster of two *pdf*-expressing cells and two *fmrf*-expressing cells is present symmetrically in the lateral regions of each optic lobe. These *fmrf*-expressing cells and *pdf*-expressing cells lie in close proximity to each other, but apparently do not overlap or merge, and thus are different neurons. The same was also clear in the *Drosophila* brains.

Although double-immunolabelling in insect brains using anti-crustacean β-PDH and anti-FMRFamide antibody suggested that insect PDF and FMRFamide-like peptides might co-exist in the same cells (*20–23*), the present study demonstrated non-overlapping expression of *pdf* and *fmrf* mRNA genes. We analysed previously the specificity of anti-crustacean β-PDH antibody (*19*), and evidenced that the PDF-expressing neurons are the same cells detected by *in situ* hybridization (*16*, *17*). The present results suggest possible cross-reactivity to unknown peptides of anti-FMRFamide antibody.

The mechanism of cell-specific transcriptional regulation of the *Drosophila fmrf* gene has been clearly

characterized (*38*). Transcriptional control elements required for spatial and temporal regulation of *fmrf* gene expression seem to be distributed over 8 kb of genomic DNA and several enhancer regions have been identified in the 5′ region of the *fmrf* gene. These cell-specific expression patterns presumably indicate the importance of the gene product, the multiple FaRPs in the insect nervous system, some of which perhaps function in circadian rhythms. If FMRFamide peptides were involved in the molecular mechanisms underlying circadian rhythms, they and their gene would be predicted to exhibit certain circadian prerequisites: for instance (*1*) peptides in secretary vesicles may exhibit a circadian rhythmicity; (*2*) mRNA may co-express in a neuron with *period* mRNA or (*3*) mRNA production may exhibit circadian rhythmicity. Experiments to reveal these points are in progress in our laboratory.

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REFERENCES

1. Price, D.A. and Greenberg, M.J. (1977) Structure of a molluscan cardioexcitatory neuropeptide. *Science* **197**, 670–671
2. Nichols, R., Schneuwly, S.A., and Dixon, J.E. (1988) Identification and characterization of *Drosophila* homologue to the vertebrate neuropeptide cholecystokinin. *J. Biol. Chem.* **263**, 12167–12170
3. Holman, G.M., Cool, B.J., and Nachman, R.J. (1986) Isolation, primary structure and synthesis of leucomyosuppressin, an insect neuropeptide that inhibits spontaneous contractions of the cockroach hindgut. *Comp. Biochem. Physiol.* **85C**, 329–333
4. Nichols, R. (1992) Isolation and structural characterization of *Drosophila* TDVDHVFLRFamide and FMRFamide-containing neural peptides. *J. Mol. Neurosci.* **3**, 213–218
5. Rao, K.R., Mohrherr, C.J., Riehm, J.P., and Zahnow, C.A. (1987) Primary structure of an analog of crustacean pigment-dispersing hormone from the lubber grasshopper *Romalea microptera*. *J. Biol. Chem.* **262**, 2672–2675
6. Stengl, M. and Homberg, U. (1994) Pigment-dispersing hormone-immunoreactive neurons in the cockroach *Leucophaea maderae* share properties with circadian pacemaker neurons. *J. Comp. Physiol.* [A] **175**, 203–213
7. Pyza, E. and Meinertzhagen, I.A. (1996) Neurotransmitters regulate rhythmic size changes amongst cells in the fly's optic lobe. *J. Comp. Physiol.* [A] **178**, 33–45
8. Meinertzhagen, I.A. and Pyza, E. (1996) Daily rhythms in cells of the fly's optic lobe: taking time out from the circadian clock. *Trends Neurosci.* **19**, 285–291
9. Mertens, I., Vandingenen, A., Johnson, E.C., Shafer, O.T., Li, W., Trigg, J.S., De Loof, A., Schoofs, L., and Taghert, P.H. (2005) PDF receptor signaling in *Drosophila* contributes to both circadian and geotaxic behaviors. *Neuron* **48**, 213–219
10. Petri, B. and Stengl, M. (1997) Pigment-dispersing hormone shifts the phase of the circadian pacemaker of the cockroach *Leucophaea maderae*. *J. Neurosci.* **17**, 4087–4093
11. Renn, S.C.P., Park, J.H., Rosbash, M., Hall, J.C., and Taghert, P.H. (1999) A *pdf* neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in *Drosophila*. *Cell* **99**, 791–802
12. Park, J.H., Helfrich-Föster, C., Lee, G., Liu, L., Rosbash, M., and Hall, J.C. (2000) Differential regulation of circadian pacemaker output by separate clock genes in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **97**, 3608–3613
13. Helfrich-Förster, C., Täuber, M., Park, J.H., Muhlig-Versen, M., Schneuwly, S., and Hofbauer, A. (2000) Ectopic expression of the neuropeptide pigment-dispersing factor alters behavioral rhythms in *Drosophila melanogaster*. *J. Neurosci.* **20**, 3339–3353
14. Shirasu, N., Shimohigashi, Y., Tominaga, Y., and Shimohigashi, M. (2003) Molecular cogs of the insect circadian clock. *Zool. Sci.* **20**, 947–955
15. Chuman, Y., Matsushima, A., Sato, S., Tomioka, K., Tominaga, Y., Meinertzhagen, I.A., Shimohigashi, Y., and Shimohigashi, M. (2002) cDNA cloning and nuclear localization of the circadian neuropeptide designated as pigment-dispersing factor PDF in the cricket *Gryllus bimaculatus*. *J. Biochem.* **131**, 895–903
16. Matsushima, A., Yokotani, S., Lui, X., Sumida, K., Honda, T., Sato, S., Kaneki, A., Takeda, Y., Chuman, Y., Ozaki, M., Asai, D., Nose, T., Onoue, H., Ito, Y., Tominaga, Y., Shimohigashi, Y., and Shimohigashi, M. (2003) Molecular cloning and circadian expression profile of insect neuropeptide PDF in black blowfly, *Phormia regina*. *Lett. Pept. Sci.* **10**, 419–430
17. Matsushima, A., Sato, S., Chuman, Y., Takeda, Y., Yokotani, S., Nose, T., Tominaga, Y., Shimohigashi, M., and Shimohigashi, Y. (2004) cDNA cloning of the housefly pigment-dispersing factor (PDF) precursors protein and its peptide comparison among the insect circadian neuropeptides. *J. Pept. Sci.* **10**, 82–91
18. Sato, S., Chuman, Y., Matsushima, A., Tominaga, Y., Shimohigashi, Y., and Shimohigashi, M. (2002) A circadian neuropeptide, pigment-dispersing factor – PDF, in the last summer cicada *Meimuna opalifera*: cDNA cloning and immunocytochemistry. *Zool. Sci.* **19**, 821–828
19. Honda, T., Matsushima, A., Sumida, K., Chuman, Y., Sakaguchi, K., Onoue, H., Meinertzhagen, I.A., Shimohigashi, Y., and Shimohigashi, M. (2006) Structural isoforms of the circadian neuropeptide PDF expressed in the optic lobes of the cricket *Gryllus bimaculatus*: Immunocytochemical evidence from specific monoclonal antibodies. *J. Comp. Neurol.* **499**, 404–421
20. Pyza, E. and Meinertzhagen, I.A. (2003) The regulation of circadian rhythms in the fly's visual system: involvement of FMRFamide-like neuropeptides and their relationship to pigment dispersing factor in *Musca domestica* and *Drosophila melanogaster*. *Neuropeptides* **37**, 277–289
21. Homberg, U., Davis, N.T., and Hildebrand, J.G. (1991) Peptide-immunocytochemistry of neurosecretory cells in the brain and retrocerebral complex of the sphinx moth *Manduca sexta*. *J. Comp. Neurol.* **303**, 35–52
22. Nässel, D.R., Shiga, S., Mohrherr, C.J., and Rao, K.R. (1993) Pigment-dispersing hormone-like peptide in the nervous system of the flies *Phormia* and *Drosophila*: Immunocytochemistry and partial characterization. *J. Comp. Neurol.* **331**, 183–198
23. Settembrini, B.P. and Villar, M.J. (2005) FMRFamide-like immunocytochemistry in the brain and aubesophageal ganglion of *Triatoma infestans* (Insecta: Heteroptera). Coexpression with β-pigment-dispersing hormone and small cardioactive peptide. *Cell Tissue Res.* **321**, 299–310
24. Schaefer, M., Picciotto, R., Thane, K., Kaldany, R.R., Taussing, R., and Scheller, R.H. (1985) *Aplysia* neurons express a gene encoding multiple FMRFamide neuropeptides. *Cell* **41**, 457–467

25. Schneider, L.E. and Taghert, P.H. (1988) Isolation and characterization of a *Drosophila* gene that encodes multiple neuropeptides related to Phe-Met-Arg-Phe-NH_2 (FMRFamide). *Proc. Natl. Acad. Sci. USA* **85**, 1993–1997
26. Taghert, P.H. and Schneider, L.E. (1990) Interspecific comparison of a *Drosophila* gene encoding FMRFamide-related neuropeptides. *J. Neurosci.* **10**, 1929–1942
27. Frohman, M.A., Dush, M.K., and Martin, G.R. (1988) Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proc. Natl. Acad. Sci. USA* **85**, 8998–9002
28. Duve, H., Johnsen, A.H., Sewell, J.C., Scott, A.G, Orchard, I., Rehfeild, J.F., and Thorpe, A. (1992) Isolation, structure, and activity of -Phe-Met-Arg-Phe-NH_2 neuropeptides (designated calliFMRFamides) from the blowfly *Calliphora vomitoria*. *Proc. Natl. Acad. Sci. USA* **89**, 2326–2330
29. Don, R.H., Cox, P.T., Wainwright, B.J., Baker, K., and Mattick, J.S (1991) 'Touchdown' PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Res.* **19**, 4008
30. Wilkinson, D.G. (Ed.) (1999) *In Situ Hybridization: A Practical Approach*, 2nd edn, Oxford University Press, Oxford
31. Orchard, I., Lange, A.B., and Bendena, W.G. (2001) FMRFamide-related peptides: a multifunctional family of structurally related neuropeptides in insects. *Adv. Insect Physiol.* **28**, 267–329
32. Nässel, D.R. (2002) Neuropeptides in the nervous system of *Drosophila* and other insects: multiple roles as neuromodulators and neurohormones. *Prog. Neurobiol.* **68**, 1–84
33. Kozak, M. (1999) Initiation of translation in prokaryotes and eukaryotes. *Gene* **234**, 187–208
34. Li, C., Kim, K., and Nelson, L.S. (1999) FMRFamide-related neuropeptide gene family in *Caenorhabditis elegans*. *Brain Res.* **848**, 26–34
35. Li, C., Nelson, L.S., Kim, K., Nathoo, A., and Hart, A.C. (1999) Neuropeptide gene families in the nematode *Caenorhabditis elegans*. *Ann. N.Y. Acad. Sci.* **897**, 239–252
36. Veenstra, J.A. (2000) Mono- and dibasic proteolytic cleavage sites in insect neuroendocrine peptide precursors. *Arc. Insect Biochem. Physiol.* **43**, 49–63
37. Taghert, P.H., Hewes, R.S., Park, J.H., O'Brien, M.A., Han, M., and Peck, M.E. (2001) Multiple amidated neuropeptides are required for normal circadian locomotor rhythms in *Drosophila*. *J. Neurosci.* **21**, 6673–6686
38. Benveniste, R.J. and Taghert, P.H. (1999) Cell type-specific regulatory sequences control expression of the *Drosophila* FMRF-NH_2 neuropeptide gene. *J. Neurobiol.* **38**, 507–520