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Functional characterization of a neuropeptide F-like receptor from *Drosophila melanogaster*

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Abstract

A cDNA clone encoding a seven-transmembrane domain, G-protein-coupled receptor (NPFR76F, also called GPCR60), has been isolated from *Drosophila melanogaster*. Deletion mapping showed that the gene encoding this receptor is located on the left arm of the third chromosome at position 76F. Northern blotting and whole mount *in situ* hybridization have shown that this receptor is expressed in a limited number of neurons in the central and peripheral nervous systems of embryos and adults. Analysis of the deduced amino acid sequence suggests that this receptor is related to vertebrate neuropeptide Y receptors. This *Drosophila* receptor shows 62–66% similarity and 32–34% identity to type 2 neuropeptide Y receptors cloned from a variety of vertebrate sources. Coexpression in *Xenopus* oocytes of NPFR76F with the promiscuous G-protein $G_{\alpha 16}$ showed that this receptor is activated by the vertebrate neuropeptide Y family to produce inward currents due to the activation of an endogenous oocyte calcium-dependent chloride current. Maximum receptor activation was achieved with short, putative *Drosophila* neuropeptide F peptides (Drm-sNPF-1, 2 and 2s). Neuropeptide F-like peptides in *Drosophila* have been implicated in a signalling system that modulates food response and social behaviour. The identification of this neuropeptide F-like receptor and its endogenous ligand by reverse pharmacology will facilitate genetic and behavioural studies of neuropeptide functions in *Drosophila*.

Introduction

Neuropeptide Y (NPY), first isolated from porcine brain as a 36 amino acid peptide, is the most abundant neuropeptide in the vertebrate brain. Neuropeptide Y sequences are highly conserved among species (Wahlestedt & Reis, 1993). These facts argue for an important role for this peptide throughout the animal kingdom. In vertebrates, NPY stimulates food consumption, affects blood pressure, induces anxiolysis, enhances memory retention and affects circadian rhythmicity (Zimanyi *et al.*, 1998). These effects are mediated by several receptor subtypes. Five receptor subtypes have been cloned (Y_1, Y_2, Y_4, Y_5 and Y_6) and all are members of the seven-transmembrane G-protein-coupled receptor family.

Little is known about the functional role of NPY-like peptides in invertebrates. However, neuropeptide F (NPF) peptides from invertebrates have been identified and share structural similarities with the vertebrate NPY superfamily. These may represent the invertebrate equivalents of this family (Maule *et al.*, 1995; Day & Maule, 1999). Several invertebrate NPF-like peptides, including those isolated from *Moniezia*, *Helix*, *Aplysia* and *Artioposthia*, range in size from 36 to 40 amino acids (Day & Maule, 1999). A number of related peptides from insects (*Leptinotarsa* and *Helicoverpa*) (Spittaels *et al.*, 1996; Huang *et al.*, 1998) and from *Loligo* and *Limulus* (Smart *et al.*, 1992; Gaus *et al.*, 1993) are much shorter (6–11 amino acids) but all have a conserved (A/L)R(P/L)RFamide sequence at their C-terminal ends.

Neuropeptide Y-like receptors have been identified in a number of invertebrate species, including *Lymneae* (Tensen *et al.*, 1998) and *Caenorhabditis elegans* (de Bono & Bargmann, 1998). The *Lymneae* receptor is activated by a peptide with a conserved . . .PQGRFamide C-terminus. The *C. elegans* receptor is activated by endogenous FMRFamide-like peptides (Reale *et al.*, 2002).

Four protein sequences have been identified from *Drosophila* as putative NPY-like receptors (CG1147, CG7395, CG12610 and CG12955) (Hewes & Taghert, 2001). CG1147 is the receptor for the *Drosophila* long NPF peptide (Garczynski *et al.*, 2002). Another protein (CG5811) was initially proposed to encode a putative NPY-like receptor (Li *et al.*, 1992) but has now been shown to be preferentially

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activated by peptides with a conserved . . .PQGRFamide C-terminus (St-Onge *et al.*, 2000).

Here, we describe the cloning, RNA expression pattern and pharmacological characterization of the seven-transmembrane NPY-like receptor known as NPFR76F (CG7395) from *Drosophila*. We demonstrate that this receptor is specifically activated by short insect NPF-like peptides when expressed in *Xenopus* oocytes in the presence of the promiscuous G-protein, $G_{\alpha 16}$ (Milligan *et al.*, 1996; Stables *et al.*, 1997). Using a reverse pharmacological approach, we show that this receptor is maximally activated by putative endogenous *Drosophila* NPF-like peptides encoded by the *sNPF* gene, which maps to chromosome position 38A7. Preliminary accounts of this work have been published in abstract form (Feng *et al.*, 1999; Hall *et al.*, 2000; Reale *et al.*, 2000). While this paper was in review, Mertens *et al.* (2002) published a pharmacological characterization of NPFR76F (which they renamed Drm-sNPF-R) expressed in Chinese hamster ovary (CHO) cells. We compare their expression results with ours in *Xenopus* oocytes.

Materials and methods

Initial polymerase chain reaction amplification of fragment GPCR60

A pair of primers (OPS3, 5′-CATAGCCCTCGACCGGTACT-3′ and OPS4, 5′-GGCAGCCAGCAGATGACGAA-3′) was designed from regions (conserved across G-protein receptor subtypes) of the *Drosophila* octopamine/tyramine receptor (Arakawa *et al.*, 1990). The forward primer, OPS3, encodes the cytoplasmic end of the third transmembrane domain of the *Drosophila* octopamine/tyramine receptor. This primer includes the DRY amino acid sequence that is found in almost all cloned G-protein-coupled receptors. The reverse primer, OPS4, encodes the middle region of transmembrane domain VI. The 100 μL polymerase chain reaction (PCR) mixture contained 0.2 mM deoxynucleotide triphosphates, 10 mM Tris-HCl buffer (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.1 μM of each primer, 2.5 U *AmpliTaq*™ DNA polymerase (Perkin Elmer Life Sciences, Boston, MA) and 300 ng *Drosophila* genomic DNA from wild-type Canton-S adult flies. Following denaturation of the substrate for 2 min at 94 °C, a reduced stringency annealing PCR was performed as follows: six cycles of 94 °C for 2 min, 33 °C for 2 min and 72 °C for 2 min followed by an additional 31 cycles with an annealing temperature of 42 instead of 33 °C. Final extension was for 10 min at 72 °C. These amplification conditions yielded five distinct products, one of which was 0.60 kb in length and was designated GPCR60.

Screening for cDNA clones

The 0.60-kb fragment (GPCR60) from the initial PCR amplification reaction was labelled with [α-^{32}P]dCTP using the Multiprime DNA labelling system (Amersham, Arlington Heights, IL, USA) and was used to screen a *Drosophila* head cDNA library in lambda-gt11 (Itoh *et al.*, 1985) (generously supplied by Dr Paul Salvaterra, Beckman Research Institute, Duarte, CA, USA). Five positive clones were identified by high stringency screening of 1×10^6 plaque-forming units. cDNA segments (~4 kb) from two clones with the largest inserts were excised with *Eco*RI and subcloned into pBluescript II SK$^+$ (Stratagene, La Jolla, CA, USA) for further analysis.

DNA sequencing

Direct sequencing of the PCR product and cDNA clone inserts was performed as described previously (Feng *et al.*, 1995, 1996; Zheng *et al.*, 1995) using the dideoxy terminator method and an ABI automated sequencer (Applied Biosystems, Foster City, CA, USA). Each segment of DNA was sequenced at least twice in both directions. The contig (GenBank Accession no. AY192578) was assembled using Geneworks software (Intelligenetics, Mountain View, CA, USA).

Northern blots

Heads, bodies and appendages (legs and antennae) were isolated from frozen adult Canton-S flies (Schmidt-Nielsen *et al.*, 1977). Poly(A)$^+$ RNA and Northern blots were prepared as described previously (Feng *et al.*, 1995; Zheng *et al.*, 1995). Poly(A)$^+$ RNA (10 μg/lane) was run on a denaturing, formaldehyde agarose (0.8%) gel and blotted onto Nytran nylon membranes (Schleicher & Schuell, Keene, NH, USA). The blots were probed with the ^{32}P-labelled 0.60 kb GPCR60 PCR fragment (10^6 cpm/mL). After high stringency washing (Feng *et al.*, 1995; Zheng *et al.*, 1995), blots were exposed to X-ray film for 24 h at −70 °C. Blots were stripped and reprobed with a cDNA probe encoding a *Drosophila* ribosomal protein (*rp49*) (O'Connell & Rosbash, 1984) to control for differences in RNA loading between lanes.

Chromosome mapping

Salivary glands were dissected from late third instar larvae grown at 21 °C and chromosome squashes prepared as described by Engels *et al.* (1985). *In situ* hybridizations to salivary gland chromosomes were done with either biotinylated double-stranded DNA probes (Engels *et al.*, 1985; Murtagh *et al.*, 1993) or with digoxigenin-labelled, single-stranded cRNA probes prepared with a digoxigenin RNA labelling kit (1175025; Roche). Pre-hybridization and hybridization of the RNA probes were done as described by Pardue (1986) except that the chromosomes were not acetylated and the hybridization solution was 50% deionized formamide, 2× SSC [sodium chloride, sodium citrate, pH 7 (Sambrook *et al.*, 1989)], 500 μg/mL sonicated, denatured salmon sperm DNA and ~100 ng digoxigenin-labelled cRNA probe. Digoxigenin probe hybridization sites were detected with alkaline phosphatase-conjugated antidigoxigenin antibody (1093274; Roche) using a colour reaction with 5-bromo-4-chlor-3-indolyl phosphate and nitro-blue tetrazolium (1681451; Roche).

Transcript expression patterns

Digoxigenin-labelled antisense RNA probes were made with a digoxigenin-RNA labelling kit (1175025; Roche) according to the manufacturer's instructions. Briefly, the 20 μL reaction mixture containing 1 μg of DNA template (pBluescript-NPFR76F cDNA cut with *Not*I), 2 μL 10× nucleotide triphosphate mix (10 mM ATP, 10 mM CTP, 10 mM GTP, 6.5 mM UTP and 3.5 mM digoxigenin-UTP), 2 μL 10× transcription buffer, 1 μL RNase inhibitor (20 U) and 2 μL T7 RNA polymerase (40 U) was incubated for 2 h at 37 °C. The reaction was terminated with 2 μL of 0.2 M EDTA. The digoxigenin-labelled RNA was mixed with 80 μL hybridization buffer [50% formamide, 5× SSC (Sambrook *et al.*, 1989), 100 μg/mL sonicated salmon sperm DNA and 50 μg/mL heparin] and stored at −20 °C.

The protocol for RNA localization was a modification of the whole-mount *in situ* hybridization method of Tautz & Pfeifle (1989). Briefly, 0–6-h embryos were aged for 18 h at 25 °C to enrich for late stage embryos. The chorion was removed by treatment with 50% sodium hypochlorite. Embryos were washed thoroughly, fixed with 10% formaldehyde and washed again with phosphate-buffered saline containing 0.1% Tween 20. The vitelline membrane was removed by incubation in heptane followed by methanol washes. The embryos were incubated in proteinase K (20 μg/mL), hybridized at 55 °C with the digoxigenin-labelled antisense RNA probe in hybridization solution and thoroughly washed with phosphate-buffered saline containing 0.1% Tween 20. The hybridized whole

mounts were incubated with pre-absorbed alkaline phosphatase-conjugated antidigoxigenin antibody and hybridization was detected as a blue precipitate following incubation with nitro-blue tetrazolium (75 μg/mL) and 5-bromo-4-chlor-3-indolyl phosphate (50 μg/mL).

Expression in Xenopus oocytes

Sense cRNA was prepared *in vitro* from the NPFR76F clone in pcDNA3.1/v5/His-TOPO (Invitrogen, The Netherlands) using the mCAP™ RNA Capping Kit (Stratagene). RNA transcripts were synthesized using T7 polymerase (Stratagene) after linearizing the plasmid with *Pme*I (New England Biolabs, Beverly, MA). T7 RNA transcripts synthesized *in vitro* with the mCAP™ RNA Capping Kit were initiated with the 5′ 7MeGpppG 5′-cap analogue. Sense cRNA was prepared in a similar manner from the $G_{\alpha 16}$ clone in pCIH1 (kindly donated by Dr Stephen Rees, Glaxo Wellcome, Stevenage, UK) after linearizing the plasmid with *Bst*XI (Promega, Madison, WI, USA) and blunting the 3′ overhangs with T4 DNA polymerase (Promega).

All experiments with *Xenopus laevis* were carried out under a Home Office (UK) project licence. Mature adult female *X. laevis* (Blades Biologicals, Edenbridge, UK) were anaesthetized by immersion in fresh water containing 1 mg/mL Tricaine. After making a small incision in the abdomen, some pieces of ovary were removed. Stage V and VI oocytes from virgin female adult *X. laevis* were manually separated and placed in sterile ND96 medium containing (in mM): NaCl, 96; KCl, 2; $CaCl_2$, 1.8; $MgCl_2$, 1; HEPES buffer (pH 7.6), 5 (supplemented with 2.4 mM sodium pyruvate, 100 U/mL penicillin, 0.1 mg/mL streptomycin and 0.2 mg/mL gentamycin). The oocytes were defolliculated enzymatically by incubation in ND96 containing collagenase (2 mg/mL) for 30 min. Oocytes were injected with 50 ng of *Drosophila* GPCR60 receptor sense cRNA, either alone or together with 50 ng of $G_{\alpha 16}$ sense cRNA (Milligan *et al.*, 1996; Stables *et al.*, 1997) and incubated at 19 °C for 2–5 d. Uninjected oocytes were used as controls. Recordings were made from oocytes using a two-microelectrode voltage-clamp technique with a −60 mV holding potential (Van Renterghem *et al.*, 1987). Oocytes were continuously superfused with ND96 during the experiments at room temperature and drugs were added to the superfusate. pEC50s were obtained using GRAPHPAD software.

Drugs

The drugs used in the classification of the expressed receptor were obtained from the following sources. Dopamine hydrochloride, DL-octopamine hydrochloride, tyramine hydrochloride, NPY porcine, peptide YY porcine, pancreatic polypeptide bovine, FMRFamide, substance P, proctolin, F-8-F-NH_2, adipokinetic hormone II (*Schistocerca*), cholecystokinin (CCK)26-33amide and Met-enkephalin were obtained from Sigma-Aldrich (Poole, Dorset, UK); NPF, lobster F1 and *Achatina* cardioexcitatory peptide 1 were obtained from Bachem (UK) Ltd. (St. Helens, Merseyside, UK). All other peptides were synthesized by the Babraham Institute Microchemical Facility.

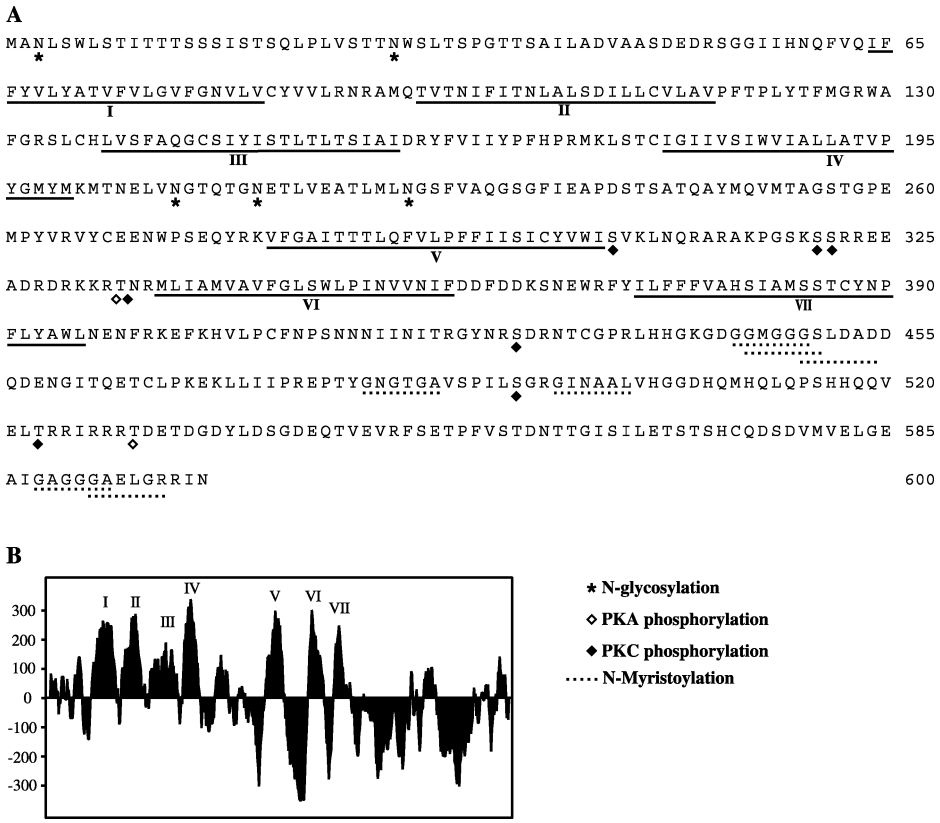

FIG. 1. The *Drosophila* neuropeptide F-like receptor (NPFR76F) sequence. (A) Deduced amino acid sequence of the *Drosophila* NPFR76F receptor (GenBank Accession no. AY192578). The proposed transmembrane domains are underlined. Consensus N-glycosylation sites (N X S/T) (★); possible protein kinase A phosphorylation motifs (R/K XX S/T) (◇); protein kinase C motifs (S/T X R/K) (◆). Possible myristoylation sites (Towler *et al.*, 1988) in the carboxy terminal tail are indicated by dashed lines under the amino acids. (B) Hydropathy plot of the deduced NPFR76F amino acid sequence. The hydropathy plot was made by the method of Kyte & Doolittle (1982) using the GCG program from the Wisconsin Genetics Computer Group software (Devereux *et al.*, 1984). Regions above zero on the *Y*-axis represent hydrophobic segments. Each of the seven hydrophobic transmembrane domains is numbered (I–VII).

Results

Cloning of NPFR76F (aka GPCR60)

To identify new G-protein-coupled receptors in *Drosophila* for further genetic manipulation, we used low stringency PCR with primers from regions conserved in all biogenic amine receptors. Initially, five fragments (1.7, 1.0, 0.68, 0.60 and 0.46 kb) were amplified from *Drosophila* genomic DNA. Direct sequencing of the PCR products revealed that the 1.7, 0.68 and 0.60-kb fragments encoded G-protein-coupled receptors. We have shown previously that the 0.68-kb fragment belongs to a novel dopamine D1-like receptor (Feng *et al.*, 1996; Reale *et al.*, 1997). The 1.7-kb fragment was a portion of the octopamine/tyramine receptor gene (Arakawa *et al.*, 1990) containing an intron. The 0.60-kb PCR fragment was used to isolate cDNA clones encoding a new G-protein-coupled receptor (GPCR60/NPFR76F/Drm-sNPF-R).

NPFR76F/GPCR60 encodes a novel Drosophila *neuropeptide Y-like receptor*

Sequencing the two largest cDNAs revealed an open reading frame encoding 600 amino acids with a predicted molecular weight of 66.5 kDa (Fig. 1A). Neither cDNA clone contained a polyA sequence. The translation initiation site was defined by the first in-frame ATG preceded by several in-frame stop codons. Its flanking sequence (AAAG) is a good match with the *Drosophila* consensus sequence for a translation start site: (A/C)AA(A/C) (Cavener, 1987). These cDNAs (originally referred to as GPCR60) were renamed NPFR76F, designating a *n*euro*p*eptide *F r*eceptor mapping to chromosome region *76F*. This name is used in the *Drosophila* genome database and in Gadfly. Other names for this gene in the *Drosophila* genome database are CG7395 and CT22771. While this manuscript was in review, Mertens *et al.* (2002) also characterized the NPFR76F receptor which they named Drm-sNPF-R.

The deduced amino acid sequence of NPFR76F shows several standard features of G-protein-coupled receptors. It has seven transmembrane domains as revealed by hydropathy analysis (Fig. 1B). As shown in Fig. 1A, it also contains the highly conserved DRY sequence immediately following the third transmembrane domain. This amino acid triplet is implicated in interaction of the receptor with G-proteins. Two potential N-linked glycosylation sites (indicated by stars in Fig. 1A) are found in the N-terminal extracellular domain preceding the first transmembrane domain and three more sites are found in the extracellular domain between transmembrane domains IV and V. Several serine and threonine residues are found in the third cytoplasmic loop and C-terminal cytoplasmic tail that are potential phosphorylation sites by protein kinase A (open diamonds in Fig. 1A) and protein kinase C (closed diamonds in Fig. 1A). In addition, in the intracellular C-terminal domain, there are four areas that are potential N-myristoylation sites (indicated by dotted lines beneath the sequence in Fig. 1A). Many G-protein-coupled receptors, such as beta-adrenergic receptors, are palmitoylated in the C-terminus (Ross, 1995). This palmitoyl group serves to anchor the receptor in the membrane and create an additional intracellular loop. It is possible that, in *Drosophila*, the myristoyl group serves the same anchor function.

Sequence comparisons with other cloned G-protein-coupled receptors in the database revealed that NPFR76F falls in generally with the neuropeptide receptors (Fig. 2). Like other neuropeptide receptors, the third intracellular loop of NPFR76F is shorter than that of other classes of G-protein-coupled receptors, including the *Drosophila* dopamine receptor (DopR99B) which was originally identified by the same method of reduced stringency PCR used here. NPFR76F shows the highest sequence conservation (60% identity, 80% similarity) with a

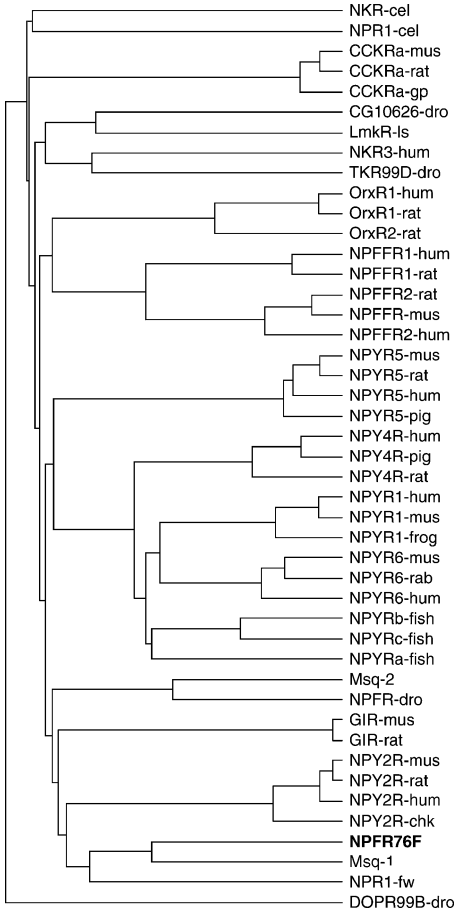

FIG. 2. Dendrogram showing the sequence similarity between the NPFR76F receptor and the most closely related G-protein-coupled receptors. The deduced amino acid sequence of NPFR76F was used in a BLAST search of the GenBank nonredundant database. Sequence alignment and dendrogram production were done with the PILEUP program of the Wisconsin Genetics Computer Group software (Devereux *et al.*, 1984). The abbreviations used for receptor sources are: cel, *Caenorhabditis elegans*; chk, chicken; dro, *Drosophila melanogaster*; frog, *Xenopus laevis*; fw, flatworm; gp, guinea pig; hum, human; ls, *Lymnaea stagnalis*; Msq, *Anopheles gambiae*; mus, mouse; rab, rabbit. The identities and accession nos. of the receptor sequences shown are: CCKRa-mus cholecystokinin A (BC020534); CCKRa-rat cholecystokinin A (D50608); CCKRa-gp cholecystokinin A (2004206A); CG10626-dro CG10626 gene product (AAF50775); DOPR99B-dro dopamine type 2 (NP524548); GIR-mus glucocorticoid-induced receptor precursor, short form RP23 (B40470); GIR-rat glucocorticoid-induced (AY029071); LmkR-ls lymnokinin (U84499); Msq-1 and Msq-2 mosquito genome sequence 1 (EAA00213) and 2 (EAA08566); NKR-cel neurokinin-like (NM077324); NKR3-hum neurokinin-3 (AAA36366); NPFFR-mus neuropeptide NPFF (AF330054); NPFFR1-hum neuropeptide FF1 (AF268898); NPFFR1-rat neuropeptide FF1 (AF268901); NPFFR2-hum neuropeptide FF2 (AF268899); NPFFR2-rat neuropeptide FF2 (AF268900); NPFR-dro neuropeptide F (AF364400); NPFR76F-dro short neuropeptide F (AY192578); NPR1-cel neuropeptide 1 (NM076415); NPR1-fw neuropeptide 1 (AF329279); NPYR1-frog NPY/PPY Y1 (L25416); NPYR1-hum neuropeptide Y1 (P25929); NPYR1-mus neuropeptide Y1 (Q04573); NPYR2-rat neuropeptide Y/YY Y2 (AY004257); NPYR2-chk neuropeptide Y2 (AF309091); NPYR2-hum neuropeptide Y2 (P49146); NPYR2-mus neuropeptide Y2 (D86238); NPYR4-pig neuropeptide Y4 (AF227955); NPYR4-rat neuropeptide Y4 (U84245); NPYR4-hum neuropeptide Y4 (P50391); NPYR5-hum neuropeptide Y5 (Q15761); NPYR5-mus neuropeptide Y5 (AAB81829); NPYR5-pig neuropeptide Y5 (O97969); NPYR5-rat neuropeptide Y5 (Q63634); NPYR6-hum neuropeptide Y6 (BAA13103); NPYR6-mus neuropeptide Y6 (U58367); NPYR6-rab neuropeptide Y6 (BAA13104); NPYRa-fish neuropeptide Ya (AAC41276); NPYRb-fish neuropeptide Yb (AF030245); NPYRc-fish neuropeptide Yc (AAC41277); OrxR1-hum orexin 1 (AAG28020); OrxR1-rat orexin 1 (AF041244); OrxR2-rat orexin 2 (AF041246); TKR99D-dro Takr99D gene product (AAF56979).

TABLE 1. A comparison of the effectiveness of different peptides in inducing inward currents in oocytes expressing the NPFR76F and $G_{\alpha16}$ clones

Peptide type	Agonist sequence	Length of sequence	Inward current response (% ± SEM)*	Oocytes tested (*n*)
NPF-like peptides				
NPF *Leptinotarsa* I	ARGPQLRLRFamide	10	100	
NPF *Leptinotarsa* II	APSLRLRFamide	8	17.0 ± 8.0	4
NPF *Moniezia*	. . .NEYFAIIGRPRFamide	39	23.0 ± 6.6	3
NPY-like peptides				
NPY porcine	. . .RHYINLITRQRYamide	36	15.7 ± 5.2	3
PYY porcine	. . .RHYLNLVTRQRYamide	36	16.2 ± 5.5	3
PP bovine	. . .RRYINMLTRPRYamide	36	1.7 ± 1.7	3
FMRFamide-like peptides				
lobster F1	TNRNFLRFamide	8	31.5 ± 9.2	10
FMRFamide	FMRFamide	4	15.8 ± 9.8	5
Drosophila myosuppressin	TDVDHVFLRFamide	10	4.8 ± 4.8	3
Drosophila FMRFamide	DPKQDFMRFamide	9	0	4
F-8-F-NH_2	FLFQPQRFamide	8	0	5
Other peptides				
substance P	RPKPQQFFGLMamide	11	13.6 ± 6.9	6
ACEP1	SGQSWRPQGRFamide	11	25.3 ± 2.4	3
SCP_B	MNYLAFPRMamide	9	12.5 ± 8.4	5
Drosophila PDH	NSELINSLLSLPKNMNDAamide	18	0	3
proctolin	RYLPT	5	0	3
adipokinetic (*Schistocerca*) II	QLNFSTGWamide	8	0	2
CCK_{26-33}amide	NY(SO_4)MGWMDFamide	8	0	3
Met-enkephalin	YGGFM	5	0	4

Agonists were applied as 2-min pulses of a 1-μM solution. *The inward currents are expressed as the percentage ±SEM of the response of the same oocyte to a control 1-μM pulse of neuropeptide F (NPF) *Leptinotarsa* I (ARGPQLRLRFamide), and the mean response to a 2-min pulse of NPF *Leptinotarsa* I was 40.6 ± 3.3 nA ($n = 107$). The biogenic amines octopamine, tyramine and dopamine generated no inward currents when tested at 1 μM ($n = 3$). *n*, No. of oocytes tested; PYY, peptide YY; PP, pancreatic polypeptide; CCK, cholecystokinin; PDH, pigment dispersing hormone; ACEP1, *Achatina* cardioexcitatory peptide; SCP_B, small cardioactive peptide b; F-8-F-NH_2, a vertebrate FMRFamide-like neuropeptide; NPY, neuropeptide Y.

G-protein-coupled receptor from mosquito (Msq-1; GenBank Accession no. EAA00213), which is likely to be the functional equivalent in the mosquito (*Anopheles gambiae*). In addition, a neuropeptide-like receptor (NPR1-fw; GenBank AF329279) from the flatworm (*Girardia tigrina*) also shows a high sequence similarity (40% identity, 68% similarity) to NPFR76F and is probably the orthologue in that organism.

Among mammalian G-protein-coupled receptors, NPFR76F groups with the NPY type 2 receptors from mouse, rat, human and chicken (32–34% identity, 62–66% similarity). The vertebrate NPY type 6 receptor grouping also shows high sequence similarity (32–33% identity, 60–61% similarity) with NPFR76F. The grouping of neuropeptide NPFR76F is distinct from other neuropeptide G-protein-coupled receptors from *Drosophila* including NPFR-dro (Garczynski *et al.*, 2002) (31% identity, 62% similarity) and CG10626 (Hewes & Taghert, 2001) (27% identity, 60% similarity).

Expression studies in Xenopus *oocytes*

To define which ligands activate the NPFR76F receptor, we expressed its cRNA alone in *Xenopus* oocytes and tested the ability of a variety of neuropeptides to initiate responses. Initially, we were unsuccessful in initiating any responses either to vertebrate NPY or to a wide range of related peptides from both vertebrates and invertebrates. Coexpression of cRNA for the promiscuous G-protein, $G_{\alpha16}$ (Milligan *et al.*, 1996; Stables *et al.*, 1997), leads to the activation of phospholipase C by receptors which do not usually couple to this enzyme in some clonal cell lines. Use of this coupling method allows a functional definition of receptor pharmacology prior to defining the physiological downstream actions of the receptor. Thus, this method is very useful for identifying the natural ligands for orphan receptors. When we coexpressed $G_{\alpha16}$ with NPFR76R, we observed that vertebrate NPY-related peptides led to small, transient inward currents in injected oocytes (Table 1). The responses had a reversal potential of −21.0 ± 3.2 mV ($n = 4$) estimated from current/voltage (I/V) plots, consistent with these responses being due to activation of the endogenous, inward calcium-dependent chloride current of the oocyte. This inward current is presumably generated by the same mechanism as originally reported by Masu *et al.* (1987) after stimulation of the bovine substance K receptor expressed in oocytes. Oocytes injected with $G_{\alpha16}$ alone and uninjected, control oocytes showed no responses (data not shown).

To determine what other peptides would stimulate currents in *Xenopus* oocytes expressing NPFR76F, we tested additional vertebrate and invertebrate neuropeptides (Table 1). Seven of these peptides showed no activity on NPFR76F (Table 1). The largest inward currents we observed followed exposure to a short NPF-like sequence reported from *Leptinotarsa* (ARGPQLRLRFamide; NPF *Leptinotarsa* I) (Spittaels *et al.*, 1996). The responses to this peptide were dose dependent (Fig. 3A and B). Threshold responses occurred between 1 and 10 nM and maximal effects were observed at 1 μM. At doses of 100 nM and above, the peptide responses desensitized before the end of the 2-min pulse.

A second *Leptinotarsa* short NPF-like sequence (APSLRLRFamide) was much less effective, as was the original long NPF sequence described from *Moniezia* (Maule *et al.*, 1992) (Table 1). As the insect NPF-like peptides all have a –RFamide C-terminus, we also tested the ability of FMRFamide-like peptides to activate the receptor (Table 1). None of the peptides tested, including two endogenous *Drosophila* N-terminally extended FMRFamide-like peptides, were more potent than the *Leptinotarsa* NPF-like peptide, ARGPQLRLRFamide. Similarly, NPFR76F was not activated by F-8-F-NH_2, a vertebrate

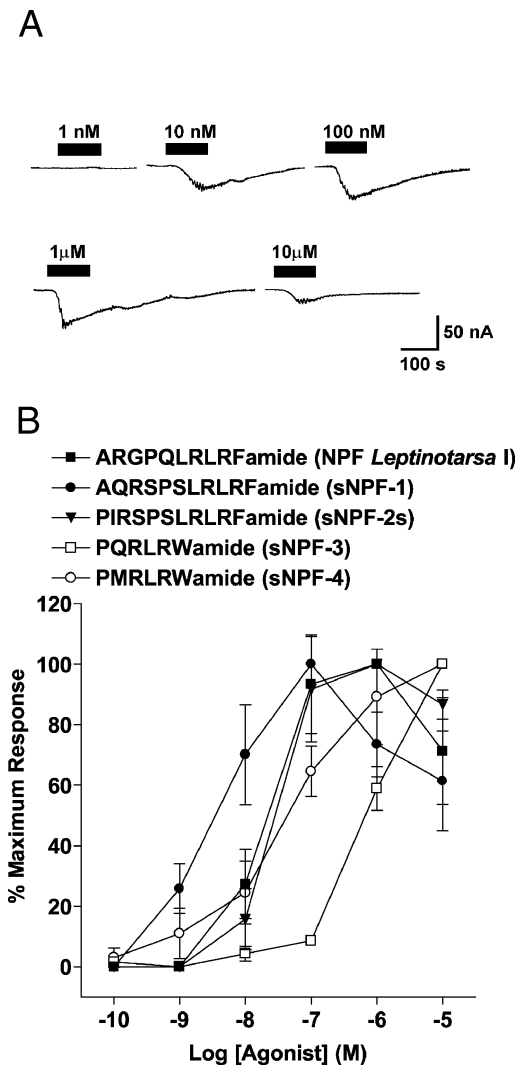

FIG. 3. (A) Typical inward current responses from a single oocyte to various concentrations of neuropeptide F (NPF) *Leptinotarsa* I (ARGPQLRLRFamide) mediated by the NPFR76F receptor coexpressed in *Xenopus* oocytes with $G_{\alpha 16}$. Two-min pulses of different concentrations of the peptide were applied to *Xenopus* oocytes 5 days after the coinjection of cRNAs. (B) Dose–response curves for NPF *Leptinotarsa* I and for putative endogenous *Drosophila* short NPF-like peptides on inward currents mediated by the NPFR76F receptor expressed in *Xenopus* oocytes. Agonists were tested as 2-min pulses at various concentrations. The inward currents generated are expressed as a percentage of the maximal response obtained for a given peptide ±SEM (n = at least 4 oocytes for each point).

endogenous FMRFamide-like neuropeptide (Yang *et al.*, 1985). As a NPY-like receptor has been cloned from *Lymnaea* (Tensen *et al.*, 1998), we tested *Achatina* cardioexcitatory peptide 1 (SGQSWRPQGRFamide) (Fujimoto *et al.*, 1990), which is closely related to the endogenous activator of the *Lymnaea* receptor. Although *Achatina* cardioexcitatory peptide 1 showed some activity (Table 1), it was far less active than ARGPQLRLRFamide.

Actions of putative endogenous Drosophila *neuropeptide F-like peptides*

As the NPFR76F receptor was maximally activated by a short insect NPF-like sequence from *Leptinotarsa*, we used genome mining to search for *Drosophila* peptides which might be functionally equivalent to (or better than) the *Leptinotarsa* I peptide at activating NPFR76F. Initially, we identified one precursor sequence, a gene (*npf*) encoding *Drosophila* NPF at chromosome location 89D3 (Table 2). We found this gene by blasting an incomplete version of the *Drosophila* genome (October 1999) with the amino acid sequence of *Aplysia* NPY (Rajpara *et al.*, 1992). This revealed a precursor molecule encoding a 36 amino acid peptide with sequence similarity to NPF. We have subsequently searched the completed *Drosophila* genome sequence and have not found any other potential NPF precursors. This same precursor (AF1178896) was identified by Brown *et al.* (1999). As this peptide (NPF-A1, Table 2) contained a potential dibasic amino acid cleavage site within its sequence, we synthesized both the shorter 28 amino acid form (NPF-A2) and the full-length peptide (NPF-A1) for testing.

A second open reading frame in the *Drosophila* genome encodes a precursor peptide for two short NPF-like peptides (Drm-sNPF-1, AQRSPSLRLRFamide and Drm-sNPF-2, WFGDVNQKPIRSPSLRLRFamide). The precursor for these peptides is encoded by the short NPF precursor (*sNPF*) gene (CG13968) (Table 2) and maps to position 38A7 on the left arm of *Drosophila* chromosome 2 (Hewes & Taghert, 2001; Vanden Broeck, 2001). The WFGDVNQKPIRSPSLRLRFamide peptide (Drm-sNPF-2) contains a potential single basic amino acid-processing site. We synthesized this peptide (Drm-sNPF-2), its shorter form (peptide Drm-sNPF-2 s, PIRSPSLRLRFamide) and the Drm-sNPF-1 peptide (all encoded by the *sNPF* gene). The same precursor was predicted (Vanden Broeck, 2001) to include the sequences for two other short peptides, PQRLRWamide and PMRLRWamide, which have been designated Drm-sNPF-3 and Drm-sNPF-4, respectively. We also synthesized and tested these peptides.

Table 2 shows that, when tested at 1 μM, the shorter NPF-like peptides derived from the *sNPF* gene (peptides Drm-sNPF-1 and Drm-sNPF-2) were more effective than the original *Leptinotarsa* I NPF-like sequence at inducing inward currents in *Xenopus* oocytes expressing NPFR76F and $G_{\alpha 16}$. Shortening of peptide Drm-sNPF-2 to the Drm-sNPF-2s form may slightly increase its effectiveness. The longer *Drosophila* peptides derived from the precursor gene *npf* at 89D3 were much less effective than the original *Leptinotarsa* I NPF-like sequence at inducing inward currents (Table 2). In addition, the PQRLRWamide (Drm-sNPF-3) and PMRLRWamide (Drm-sNPF4) peptides were much less effective than the original *Leptinotarsa* I sequence at inducing inward currents (Table 2).

Dose–response curves for the shorter endogenous *Drosophila* NPF-like peptides encoded by the *sNPF* gene at 38A7 (Fig. 3B) reveal that AQRSPSLRLRFamide (peptide Drm-sNPF-1) was the most potent peptide tested ($pEC50 = -8.84 \pm 0.47$, $n = 3$). It showed a threshold for the generation of inward currents between 100 pM and 1 nM and a maximal effect at 100 nM. The second putative endogenous *Drosophila* NPF-like peptide encoded by the *sNPF* gene, PIRSPSLRFamide (peptide Drm-sNPF-2 s) ($pEC50 = -7.62 \pm 0.26$, $n = 3$), and the original *Leptinotarsa* I NPF-like sequence, ARGPQLRLRFamide ($pEC50 = -7.83 \pm 0.35$, $n = 3$), were an order of magnitude less potent than AQRSPSLRLRFamide. These results justify the classification of NPFR76F as a NPF-like receptor and suggest that the short peptide AQRSPSLRLRFamide (sNPF-1) may be the endogenous agonist for this receptor. The other two short peptides encoded by the *sNPF* gene (Vanden Broeck, 2001), PQRLRWamide ($pEC50 = -6.1 \pm 0.04$, $n = 3$) and PMRLRWamide ($pEC50 = -7.30 \pm 0.07$, $n = 3$), were 2.7 and 1.5 orders of magnitude, respectively, less potent than the AQRSPSLRLRFamide sequence, leading us to question their designation as true short NPF-like peptides.

Gene mapping

Although these heterologous expression studies provide information about the pharmacological specificity of NPFR76F, they do not

TABLE 2. Effectiveness of putative *Drosophila* neuropeptide-F-like peptides in inducing inward currents in oocytes expressing NPFR76F and $G_{\alpha 16}$

Gene	Peptide sequence	Amino acids (*n*)	Inward current response (% ± SEM)*	Oocytes tested (*n*)
GENE *npf* at 89D3				
(aka *CG10342*, *dNPF*, *Drm-NPF* and *NP-PP*)				
NPF89D3: Peptide NPF-A1:	SNSRPPRKNDVNTMADAYKFLQDLD TYYGDRARVRFamide	36	17.4 ± 7.8	6
NPF89D3: Peptide NPF-A2:	NDVNTMADAYKFLQDLDTYYGDRA RVRFamide	28	27.1 ± 12.6	4
GENE *sNPF* at 38A7				
(aka *CG13968*, *Drm-sNPF*, *Drm-sNPF-1*, *Drm-sNPF-2*, *Drm-sNPF-3*, *Drm-sNPF-4*, *LRLRFamide*, *38B.2* and *short neuropeptide F precursor*)				
Peptide sNPF-1:	AQRSPSLRLRFamide	11	155.5 ± 17.2	20
Peptide sNPF-2:	WFGDVNQKPIRSPSLRLRFamide	19	123.6 ± 34.3	9
Peptide sNPF-2s:	PIRSPSLRLRFamide	11	176.2 ± 43.2	7
Peptide sNPF-3:	PQRLRWamide	6	33.3 ± 5.3	12
Peptide sNPF-4:	PMRLRWamide	6	50.2 ± 8.9	6

*Average percentage response ±SEM compared with control application of 1 μM ARGPQLRLRFamide response, and the mean response to 1 μM ARGPQLRLRFamide is 40.6 ± 3.3 nA ($n = 107$).

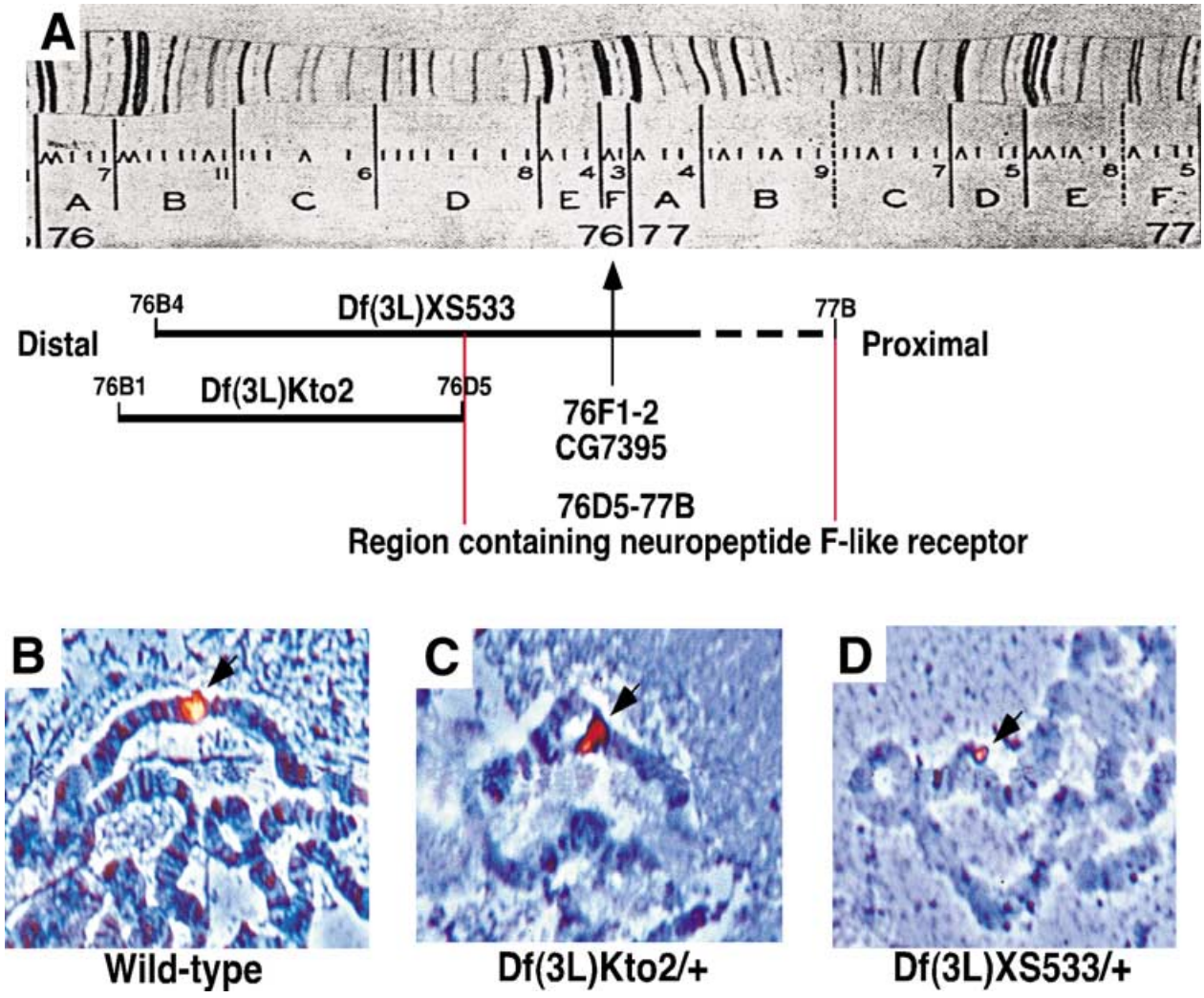

FIG. 4. *In situ* hybridization and deletion mapping of the NPFR76F receptor gene to region 76F of the *Drosophila* salivary gland chromosomes. (B–D) Arrows point to sites of hybridization of the NPFR76F probe to wild-type (B) and deletion heterozygote (C and D) salivary gland chromosomes. (A) Schematic diagram of the banding pattern of segments 76 and 77 of the left arm of *Drosophila* salivary gland chromosome 3 where the NPFR76F gene maps. The regions missing in the deletions are shown as solid lines beneath the schematic diagram. The dashed line on the proximal side of deletion DF(3L)XS533 designates a region of uncertainty with respect to the end of this deletion. Distal and proximal indicate orientation relative to the centromere.

indicate what the role of this receptor is in the organism. Identification of mutants affecting the receptor is a first step in this direction. For this reason we have used *in situ* hybridization to salivary gland chromosomes from wild type to determine the map position of the gene encoding this receptor. We have also analysed hybridization to deletion heterozygotes to provide a basis for future identification of mutations that map to the same area and thus are candidates for mutations in *NPFR76F*. The results of these studies are summarized in Fig. 4. We found that this gene maps to region 76F1-2 on the left arm of the third chromosome (Fig. 4A and B). It maps within the *Df(3L)XS533* deletion (Fig. 4D) but is proximal to the *Df(3L)Kto2* deletion (Fig. 4C). Thus, mutations which are uncovered by the *Df(3L)XS533* deletion are candidates for mutations in this gene. These results are consistent with the localization reported in the *Drosophila* genome (Adams *et al.*, 2000).

mRNA distribution in adults

Expression of *NPFR76F* transcripts was assessed by Northern blot analysis of poly(A)$^+$ RNA prepared from adult body parts. A single transcript of 6.5 kb was detected in both heads and appendages (legs and antennae) (Fig. 5), suggesting that *NPFR76F* is expressed in both the central and peripheral nervous systems. In addition to finding transcript in heads and appendages, trace amounts were also seen in bodies. When compared with the amount of RNA loaded from each body part (as indicated by the ubiquitous *rp49* loading control), the relative abundance of transcript in bodies is very low. This distribution of the *NPFR76F* transcript is consistent with a role for this NPF-like receptor in the *Drosophila* nervous system.

NPFR76F receptor transcript expression in embryos

To further refine the NPFR76F receptor transcript expression, we used *in situ* hybridization with a digoxigenin-labelled antisense RNA probe to whole mounts of the mature embryos (Fig. 6A–E). The central nervous system in embryos is composed of two dorsal brain hemispheres and a fused ventral ganglion. The NPFR76F receptor is expressed both in the dorsal brain and in the ventral ganglion. In the brain the receptor is strongly expressed in the specific cells in the dorsal posterior region (Fig. 6A, arrow). Using camera lucida drawings of three different embryos (summarized in Fig. 6F), we estimate that there are 22–24 cells in each brain lobe expressing NPFR76F, including the strongly expressing cells. One of these strongly expressing cells is indicated by the blue arrow in Fig. 6A and F. In the ventral ganglion, pairs of cells along the ventral midline (black arrow in Fig. 6C), as well as cells found in a bilaterally symmetric pattern in a more lateral position from the midline, also express receptor mRNA (red arrows in Fig. 6C, D and F). In each full segment of the ventral ganglion, the receptor is strongly expressed in eight to 12 cells, including a pair of cells at the midline in each segment.

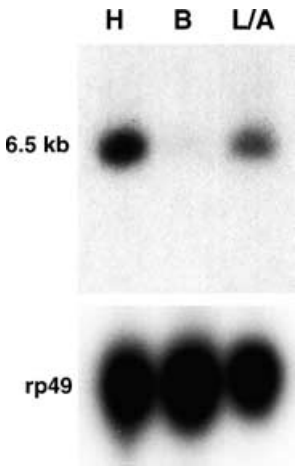

FIG. 5. Northern blot analysis of the NPFR76F receptor. A Northern blot of poly(A)$^+$ RNA isolated from heads (H), bodies (B) and legs/antennae (L/A) was probed with the ^{32}P-labelled 0.60 kb polymerase chain reaction product. To control for mRNA recovery, the blot was reprobed with ribosomal protein cDNA (*rp49*), which is expressed throughout the organism (O'Connell & Rosbash, 1984).

In the peripheral nervous system the receptor is expressed in a subset of sensilla (Fig. 6B and E, black and red arrows) and in the anterior sensory complex (Fig. 6D, black arrowhead). From the camera lucida drawings, we estimate that there are 10–14 cells in the anterior sensory complex, including the antennomaxillary complex, the labral sensory complex and the labial sensory complex, that express NPFR76F. Finally, in the posterior sensilla, there are eight cells that express NPFR76F. Figure 6F shows a composite summary of both the central and peripheral sites expressing NPFR76F in the embryo. The expression pattern of the NPFR76F receptor in many specific cells in the dorsal brain, the ventral ganglion, lateral sensilla, the anterior sensory complex and the posterior sensilla suggests that this receptor is involved in a widespread modulation of neuronal activity.

Discussion

We have identified a novel seven-transmembrane G-protein-coupled receptor from the fruitfly, *Drosophila melanogaster*. This receptor shows structural similarity to vertebrate neuropeptide receptors. We have designated this receptor NPFR76F based on its activation by insect short NPF-like peptides (sNPF-1, -2 and -2s) and its chromosomal location on the left arm of chromosome 3 at position 76F (this work and Adams *et al.*, 2000). This same receptor was identified independently while the present work was under review (Mertens *et al.*, 2002).

The *Drosophila* NPFR76F receptor is most similar to the vertebrate NPY type 2 (Y2) receptor. Like these receptors, the *Drosophila* NPFR76F receptor has cysteines in extracellular domain 2 (between transmembrane domains II and III) and in extracellular domain 3 (between transmembrane domains IV and V) which may form a disulphide bridge. In addition, NPFR76F has a conserved cysteine in the cytoplasmic tail in the region of the palmitoylation site in vertebrate Y2 receptors. However, the region surrounding this cysteine in the *Drosophila* receptor is not a typical palmitoylation consensus sequence. In addition, we propose that the several serine and threonine residues found in the third cytoplasmic loop and the intracellular C-terminal tail are possible sites for regulation by protein kinase A or protein kinase C phosphorylation.

Many vertebrate NPY type 2 receptors contain a DRH sequence at the start of the second intracellular loop. However, NPFR76F has a DRY sequence (which is found in many invertebrate NPY-like receptors) at this position. In vertebrates, the Y2 receptor is thought to be predominantly pre-synaptic and to mediate many of its effects by suppression of neurotransmitter release. For example, the release of glutamate from terminals synapsing on rat hippocampal CA1 neurons is inhibited by activation of Y2 receptors. In addition, peripheral Y2 receptors are associated with suppression of transmitter release from sympathetic and sensory C fibres (Michel *et al.*, 1998; Ingenhoven & Beck-Sickinger, 1999). Thus, it is possible that NPFR76F is also responsible for widespread control of neurotransmitter release in the *Drosophila* nervous system.

Among the vertebrate receptors, NPFR76F also shows high structural relatedness (34–35% identity, 63–64% similarity) to glucocorticoid-induced receptors from mouse and rat (GIR-mus and -rat in

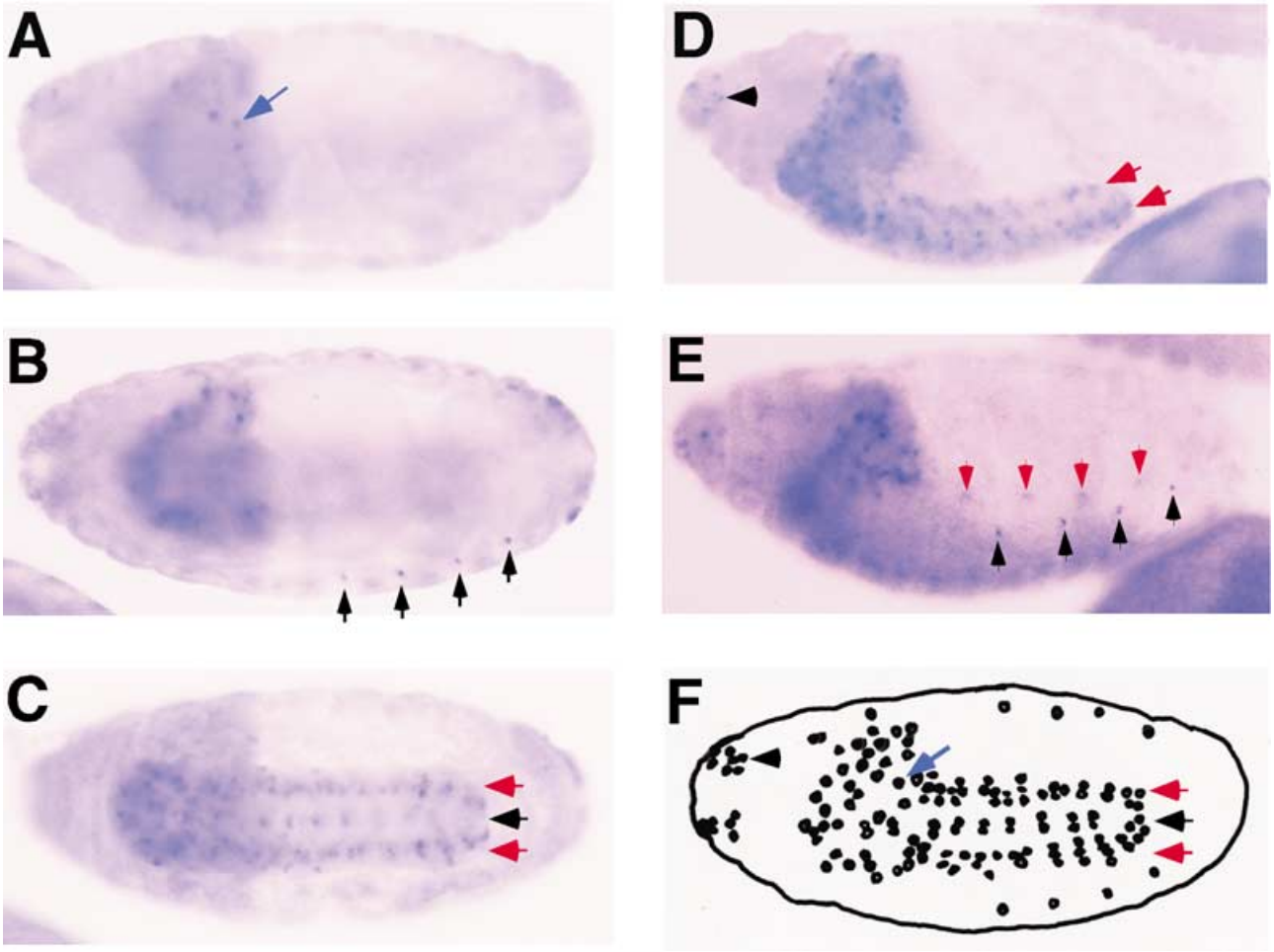

FIG. 6. NPFR76F receptor mRNA is expressed in both the central nervous system and the peripheral nervous system of *Drosophila* stage 16 embryos. The NPFR76F receptor expression was determined by *in situ* hybridization with a digoxigenin-labelled antisense RNA probe. (A and B) Dorsal views; (C) ventral view; (D and E) lateral views. (A) NPFR76F is strongly expressed in a small number of bilaterally symmetric cells in the posterior region of the cerebral hemispheres of the central nervous system. The arrow points to one of these strongly expressing cells. (B) A subset of sensilla in the peripheral nervous system express the neuropeptide-F-like receptor. The black arrows in (B and E) indicate the same set of cells from different views. (C) In the ventral ganglion, the black arrow points toward pairs of cells along the midline. The red arrows point to bilaterally symmetric receptor-expressing cells found in each segment of the ventral nerve cord. (D) A lateral view shows cells in the central nervous system (red arrows) and the anterior sensory system (black arrow) that express the NPFR76F receptor. Bilaterally symmetric pairs of cells express NPFR76F in the ventral ganglion. Red arrows point to the same sets of cells as indicated by the red arrows in (C). Black arrow points to the labial sensory complex. (E) There are four sensilla on each side that express NPFR76F. The black upward arrows indicate four cells in the plane of focus. The red downward arrows point to the four cells on the other side (seen out of focus). These sets of four cells are the same as indicated by the arrows in (B). (F) composite diagram from the dorsal perspective showing the positions of all NPFR75F-expressing cells detected in this study.

Fig. 2). These receptors may be involved in neuroadaptation to psychostimulants, such as amphetamine, and in the regulation of memory, stress and reward in limbic circuits (Wang *et al.*, 2001). The endogenous ligands for these receptors are not known (Harrigan *et al.*, 1989, 1991; Parker *et al.*, 2000). It will be interesting to determine if NPFR76F expression is also controlled by steroids and to determine if the vertebrate glucocorticoid-induced receptors can be activated by NPY- or NPF-related neuropeptides.

The NPFR76F receptor is relatively close in sequence similarity but in a distinct grouping from the cloned receptor NPFR-dro (activated by the *Drosophila* long NPF-like peptide NPF-A1; Garczynski *et al.*, 2002). Previous studies identified another receptor in *Drosophila* (NepYr; CG5811) that is activated by NPY-like sequences when expressed in *Xenopus* oocytes (Li *et al.*, 1992) but this receptor is activated preferentially by . . .PQGRFamide-like peptides (St-Onge *et al.*, 2000). In addition, a number of other putative G-protein-coupled receptors (not shown in Fig. 2) with some sequence similarity to vertebrate NPY-like receptors have been identified in the *Drosophila* genome (Adams *et al.*, 2000; Hewes & Taghert, 2001). Thus, the *Drosophila* genome appears to encode multiple receptors for NPY-like neuropeptides.

When we tried to activate the NPFR76F receptor expressed by itself in *Xenopus* oocytes using vertebrate and invertebrate NPY-like peptide sequences, we were unable to detect any induced inward currents. We attempted to boost our chances of observing pharmacologically-induced currents in *Xenopus* oocytes by coexpressing the NPFR76F receptor with the promiscuous G-protein, $G_{\alpha 16}$ (Milligan *et al.*, 1996; Stables *et al.*, 1997). $G_{\alpha 16}$ can couple G-protein-coupled receptors to phospholipase C in cell lines where these receptors are not normally coupled to this enzyme (Milligan *et al.*, 1996; Stables *et al.*, 1997). Our results show, for the first time, that this technique can be successfully applied in *Xenopus* oocytes. Using this promiscuous G-protein coexpression approach, we have demonstrated that the NPFR76F receptor can be activated by vertebrate NPY-like peptides to produce significant

TABLE 3. Sequence comparison of invertebrate neuropeptide F-like (NPF) and vertebrate neuropeptide Y-like (NPY) peptides

Name and source	Peptide sequence	Amino acids in peptide (*n*)
NPF *Aplysia*	DNSEMLAP**P**PR**P**EEF TSAQQ– –LRQ**Y**LAA**L** NE**Y**YSIMG**RP**R**F**amide	40
NPF *Helix*	STQMLSP**P**ER**P**REF RHPN**E**– –LRQ**Y**LKE**L** NE**Y**YAIMG**RT**R**F**amide	39
NPF *Moniezia*	PDKDFIVN**P**SEL VLDNKAALRD**Y**LRQI NE**Y**FAIIG**RP**R**F**amide	39
NPF *Artioposthia*	KVVHLR**P**RSS FSS**E**DEYQI**Y**LRNV SK**Y**IQLYG**RP**R**F**amide	36
NPF *Drosophila* (map position 89D3)	SNSRP**P**RKN DVNTMADAYKFLQD**L** DT**Y**YGDRA**RV**R**F**amide	36
NPY human	YPSK**P**DN**P**GED APA**E**D–MAR**Y**YSA**L** RH**Y**INLIT**RQ**R**Y**amide	36
PepYY human	YPIK**P**EA**P**GED ASP**E**E–LNR**Y**YAS**L** RH**Y**LNLVT**RQ**R**Y**amide	36
PancPP human	APLE**P**VY**P**GD NATP**E**Q–MAQ**Y**AAD**L** RR**Y**INMLT**RP**R**Y**amide	36
NPF *Helicoverpa* I	QAA**RP**R**F**amide	7
NPF *Helicoverpa* II	AA**RP**R**F**amide	6
NPF *Loligo*	YAIVA**RP**R**F**amide	9
NPF *Leptinotarsa* I	ARGPQL**RL**R**F**amide	10
Head *Aedes*	pERP<u>P</u>SLKT**RF**amide	10
NPF *Limulus*	GGRSPSL**RL**R**F**amide	11
NPF *Leptinotarsa* II	APSL**RL**R**F**amide	8
NPF *Periplaneta*	ANRSPSL**RL**R**F**amide	11
sNPF-1 *Drosophila* (map position 38A7)	AQRSPSL**RL**R**F**amide	11
sNPF-2 *Drosophila* (map position 38A7)	WFGDVNQKPIRSPSL**RL**R**F**amide	19
sNPF-3 *Drosophila* (map position 38A7)	PQ**RL**R**W**amide	6
sNPF-4 *Drosophila* (map position 38A7)	PM**RL**R**W**amide	6

In peptide sequences: pE, Pyroglutamate; <u>P</u>, hydroxyproline. Bold type indicates match.

inward currents due to calcium-dependent activation of the endogenous oocyte inward chloride current. Further, we have shown that the receptor is activated preferentially by the NPF family. The NPF family are thought to be invertebrate homologues of the vertebrate NPYs (Maule *et al.*, 1995; Day & Maule, 1999). Our initial results indicated that the NPFR76F receptor was maximally activated by the shorter members (sNPFs) of the insect NPF family (Spittaels *et al.*, 1996), in preference to longer NPF sequences (Table 3). Some of these shorter peptides from *Aedes* (Matsumoto *et al.*, 1989), *Limulus* (Gaus *et al.*, 1993) and *Periplaneta* (Veenstra & Lambrou, 1995) have been previously designated as head peptides (Huang *et al.*, 1998). However, we prefer the designation 'short NPF-like neuropeptides' (Spittaels *et al.*, 1996) in view of the activity we have demonstrated for them on the *Drosophila* NPFR76F receptor.

Drosophila express both long and short NPF-like neuropeptides. We have demonstrated that putative precursor molecules for endogenous NPF-like peptides can be identified from the *Drosophila* genome. The gene (*npf*) at 89D3 encoding the longer putative NPF peptide (NPF-A1) and its truncated form (NPF-A2) has been cloned (Brown *et al.*, 1999). We have demonstrated that these peptides show a low level of activity on the NPFR76F receptor. In contrast, we show that two shorter, putative endogenous NPF-like peptides (sNPF-1 and sNPF-2) encoded by a different gene (*sNPF*) are more potent than the longer NPF-like peptides at activating inward currents through NPFR76F.

To date, the NPFR76F receptor has been expressed in two systems (CHO cells and *Xenopus* oocytes), allowing us to now compare and contrast the results from each system. Mertens *et al.* (2002) reported that, when the receptor was expressed in a CHO cell line, sNPF-1 (AQRSPSLRLRFamide) and their variant of sNPF-2 (SPSLRLRFamide) were equipotent. However, our findings in the *Xenopus* oocyte expression system are somewhat different depending on whether efficacy or potency is considered. We find that sNPF-1 and WFGDVNQKPIRSPSLRLRFamide (which we call sNPF-2 following the original nomenclature by Vanden Broeck (2001) and which is a longer version of what Mertens *et al.* (2002) call sNPF-2), are equally efficacious at inducing inward currents in terms of the maximal inward currents generated (Table 2). Also equally efficacious at inducing inward currents in oocytes is our sNPF-2s (PIRSPSLRLRFamide), which is another longer version of the SPSLRLRFamide peptide used by Mertens *et al.* (2002). However, when we consider potencies, sNPF-1 (AQRSPSLRLRFamide) is an order of magnitude more potent than sNPF-2s (PIRSPSLRLRFamide) in terms of pEC50 values in the oocyte system (Fig. 3).

Mertens *et al.* (2002) also report that the sNPF-3 (PQRLRLWamide) and sNPF-4 (PMRLRWamide) peptides were as potent as sNPF-1 (AQRSPSLRLRFamide) and SPSLRLRFamide at activating the NPFR76F in CHO cells. Our results with the *Xenopus* oocyte system strongly disagree with this conclusion. In the oocyte expression system, sNPF-3 (PQRLRWamide) and sNPF-4 (PMRLRWamide) were substantially less effective (Table 2) as well as being 2.74 and 1.54 orders of magnitude less potent, respectively, than sNPF1 (AQRSPSLRLRFamide) (Fig. 3B). We do not know the reason for the discrepancies between our results and those of Mertens *et al.* (2002), but it might reflect differences in the sensitivity of the assay systems used in the two different expression systems. Alternatively, the relative potency of the different agonists tested may depend on the relative levels of $G_{\alpha 16}$ expressed in the different systems.

A recent paper by Baggerman *et al.* (2002), published when the present work was under review, provides indirect evidence that the true sNPF-2 peptide in *Drosophila* larvae may be SPSLRLRFamide rather than the longer sequence PIRSPSLRLRFamide which we tested in the present study. Mertens *et al.* (2002) suggest that SPSLRLRFamide is as potent as AQRSPSLRLRFamide on the NPFR76F receptor expressed in CHO cells. Although we did not test SPSLRLRFamide, we did find that the longer PIRSPSLRLRFamide was less potent than AQRSPSLRLRFamide. Thus, the nature of the endogenous, physiologically active sNPF-2 peptide awaits its definitive isolation and a description of its distribution in both the larval and adult nervous systems of *Drosophila*.

The functional roles of NPF-like peptides in invertebrates are not clear at this time. We are raising antibodies against these peptides to determine their cellular locations and are using genetic approaches to determine their physiological roles in *Drosophila*. The original NPF-like molecule, isolated from the parasitic platyhelminth worm, *Moniezia expansa*, has a widespread distribution in both central and peripheral nervous systems (Maule *et al.*, 1992, 1995; Day & Maule,

1999). It may be colocalized with acetylcholine and function to modulate cholinergic signalling (Maule *et al.*, 1995). It is found predominantly in nerves that innervate muscle. In addition, high levels have been detected immunocytochemically in the reproductive apparatus of parasitic flatworms, especially in the egg-laying apparatus (Maule *et al.*, 1995). In the marine mollusc, *Apylsia*, a neuromodulatory role has also been suggested for the endogenous long NPF-like peptide. It has inhibitory effects on the activity of the neuroendocrine bag cell neurons (Rajpara *et al.*, 1992).

In insects, NPF-like neuropeptides are likely to function as neurotransmitters or neuromodulators. These peptides may also have endocrine signalling functions associated with the gut (Huang *et al.*, 1998; Garczynski *et al.*, 2002). Preliminary immunohistochemical analysis indicates that, just like vertebrate NPY, the insect NPF-like peptides have a widespread occurrence in both the insect central nervous system and within paraneurons of the mesenteric epithelium (Remy *et al.*, 1988; Schoofs *et al.*, 1988; Verhaert *et al.*, 1993, 1994; Spittaels *et al.*, 1996). The long NPF-like peptide sequence NPF-A1 in *Drosophila* (Brown *et al.*, 1999) has been shown to activate a G-protein-coupled receptor (Garczynski *et al.*, 2002) that is distinct from the receptor described in the present work. Further, the precursor molecule for the larger endogenous NPF-like peptide in *Drosophila* has been shown by both immunocytochemistry and *in situ* hybridization to be expressed in many neurons and in midgut endocrine cells in larvae and adults (Brown *et al.*, 1999). Both techniques revealed the presence of this peptide in two pairs of cells in the protocerebral region of both larval and adult brains. However, these immunocytochemical studies could be detecting both the long and the short forms of insect NPF-like peptides as the antibodies used were raised against the conserved C-terminal region of these peptides (Huang *et al.*, 1998). In *Helicoverpa* an endocrine role for the release of short NPF-like peptides from the midgut has been suggested (Huang *et al.*, 1998). The two *Leptinotarsa* short NPF-like peptides, shown in our studies to activate NPFR76F, affect ovarian development in *Locusta migratoria* and *Neobellieria bullata* (Cerstiaens *et al.*, 1999). However, it is not known if these are direct actions of the peptides (Orchard *et al.*, 2001).

Several lines of evidence indicate an important role for NPY-like peptides in the control of feeding behaviour. Neuropeptide Y in vertebrates, for instance, is the most potent stimulator of food intake known (Zimanyi *et al.*, 1998). Changes in the expression level of the long *Drosophila* NPF peptide NPF-A1 have been correlated with gustatory exposure to sugar. This has led investigators to suggest that peptide NPF-A1 may be involved in control of insect feeding behaviour (Shen & Cai, 2001). In addition, one of the cloned NPY-like receptors from *C. elegans* (NPR-1) (de Bono & Bargmann, 1998), which is expressed in neurons in the head, ventral nerve cord and preanal ganglion, has an important role in switching the animal between solitary and social feeding behaviour. A loss-of-function mutant in this receptor converts a solitary strain to social feeding behaviour.

Our identification, using reverse pharmacology, of potent putative endogenous neuropeptide activators of the novel NPFR76F receptor will allow the analysis of the physiological role of this novel G-protein-coupled neuropeptide receptor. At the present time we do not know if both the long (NPF) and short (sNPF) forms of NPF-like peptides in *Drosophila* are involved in the control of feeding. Future studies using mutations in the NPFR76F receptor will help to resolve this question and may reveal other roles for this receptor in *Drosophila*.

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Abbreviations

CHO, Chinese hamster ovary; NPF, neuropeptide F; NPY, neuropeptide Y; PCR, polymerase chain reaction.

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