Reverse physiology in *Drosophila*: identification of a novel allatostatin-like neuropeptide and its cognate receptor structurally related to the mammalian somatostatin/galanin/opioid receptor family

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By using degenerate oligonucleotide primers deduced from the conserved regions of the mammalian somatostatin receptors, a novel G-protein-coupled receptor from *Drosophila melanogaster* has been isolated exhibiting structural similarities to mammalian somatostatin/galanin/opioid receptors. To identify the bioactive ligand, a 'reverse physiology' strategy was used whereby orphan *Drosophila* receptor-expressing frog oocytes were screened against potential ligands. Agonistic activity was electrophysiologically recorded as inward potassium currents mediated through co-expressed G-protein-gated inwardly rectifying potassium channels (GIRK). Using this approach a novel peptide was purified from *Drosophila* head extracts. Mass spectrometry revealed an octapeptide of 925 Da with a sequence Ser-Arg-Pro-Tyr-Ser-Phe-Gly-Leu-NH₂ reminiscent of insect allatostatin peptides known to control diverse functions such as juvenile hormone synthesis during metamorphosis or visceral muscle contractions. Picomolar concentrations of the synthesized octapeptide activated the cognate receptor response mediated through GIRK1, indicating that we have isolated the 394-amino-acid *Drosophila* allatostatin receptor which is coupled to the Gi/Go class of G proteins.

Keywords: allatostatin/*Drosophila*/G proteins/inwardly rectifying potassium channels/somatostatin

Introduction

The mammalian G-protein-coupled receptors (GPCRs) for neuropeptides such as the somatostatin (SST) or opioid peptides exist in many different subtypes which form their own subfamily within the larger family of type I (rhodopsin-like) GPCRs (e.g. Darlison and Richter, 1999). There are at least nine different receptor genes for which the physiological ligands are known [somatostatin receptor (SSTR)1–5; μ -, κ -, δ - and orphanin FQ-opioid receptors]; in addition several orphan receptors have been characterized where the ligands remain to be identified (O'Dowd *et al.*, 1995). It is unclear at which stage in evolution this multiplicity of subtypes arose. Previous studies have addressed this issue by characterizing opioid and SSTR genes in lower vertebrates; in the case of the bony fish

Catostomus commersoni, we have identified six partial cDNAs which are all related to the mammalian opioid receptors (Darlison *et al.*, 1997). Similarly, a cDNA coding for a SSTR has also been detected in a lower vertebrate (Siehler *et al.*, 1999). Taken together, these data suggest that multiple opioid/somatostatin-receptor like genes were present early in vertebrate evolution and may therefore have also existed before the evolution of vertebrates. However, all attempts so far have failed to detect similar genes in invertebrate species (Li *et al.*, 1996).

On the other hand, several neuropeptide receptors have been identified in insects, namely in *Drosophila melanogaster* (Li *et al.*, 1992; Monnier *et al.*, 1992; Hauser *et al.*, 1998; Vanden Broeck *et al.*, 1998). cDNAs have been cloned which code for receptors that can be activated by mammalian tachykinin peptides (Monnier *et al.*, 1992) and, albeit with low affinity, by neuropeptide Y (Li *et al.*, 1992), yet these peptides have not been detected in insects. Except for the diuretic hormone receptor of *Manduca sexta* (Reagan, 1994), seven transmembrane (TM) receptors identified from insects are only poorly characterized in terms of their natural ligands. Vice versa for most of the numerous insect neuropeptides their cognate presumably GPCRs are unknown (for a review, see Gäde *et al.*, 1997; Vanden Broeck *et al.*, 1998).

Here we report the identification of the first invertebrate receptor that exhibits structural similarities to the mammalian SST/galanin/opioid receptor family. As this receptor was not activated efficiently by any of the known mammalian neuropeptides, the natural ligand was searched for via a 'reverse physiology' (also referred to as 'reverse pharmacology'; Meunier *et al.*, 1995; Reinscheid *et al.*, 1995; Tensen *et al.*, 1998) approach by screening the functional expressed orphan *Drosophila* receptor against peptide fractions purified from *Drosophila* head extracts. The identified novel octapeptide is a member of the allatostatin (AL) peptide family controlling diverse functions including the synthesis of juvenile hormones known to play a central role in metamorphosis and reproduction in various insect species.

Results

Cloning of a Drosophila seven transmembrane receptor structurally related to the mammalian SST/galanin/opioid receptor family

In order to identify insect GPCRs that are related to mammalian SSTRs, degenerate oligonucleotide primers were designed based on DNA sequences encoding the conserved regions of the first extracellular loop (forward primer) and the sixth transmembrane domain (reverse primer) of the known mammalian SSTRs. RT-PCR experiments performed on poly(A)⁺ RNA isolated from adult *Drosophila* heads resulted in the amplification of a 507 bp

Fig. 1. Primary structure of the novel *Drosophila* seven TM receptor aligned to members of the somatostatin (rat SSTR2; Kluxen *et al.*, 1992), galanin (rat galanin1; Parker *et al.*, 1995) and opioid (rat μ opioid; Fukuda *et al.*, 1993) receptor family, using the computer program PILEUP (Wisconsin Sequence Analysis Package, Version 8, Genetics Computer Group, Madison, WI). Amino acids on black background with white letters indicate residues involved in the specificity of ligand binding (Kaupmann *et al.*, 1995; Metzger and Ferguson, 1995; Nehring *et al.*, 1995; Kask *et al.*, 1996). The putative transmembrane regions, as defined by hydropathy analysis, are boxed. Asterisks indicate residues that are identical or similar in all four receptors. The following potential sites for modifications are listed: circles, N-glycosylation; squares, protein kinase C phosphorylation; triangles, protein kinase A phosphorylation; diamonds, palmitoylation.

cDNA fragment. Sequence comparison showed that this fragment exhibited highest similarity to the mammalian SST/galanin/opioid receptor family. Using the same poly(A)⁺ RNA preparation, 5' and 3' rapid amplification of cDNA ends (RACE) experiments gave rise to a cDNA of 1826 bp; sequence analysis revealed an open reading frame of 1182 bp coding for a 43.2 kDa protein that comprises 394 amino acid residues (Figure 1). Preceded by an in-frame stop codon, the methionine codon at position 231 of the cDNA sequence was assigned as the potential translation initiation site (for the complete cDNA sequence, see accession No. AF163775 in the DDBJ/EMBL/GenBank database). Hydrophobicity analysis of the deduced amino acid sequence indicated the presence of seven hydrophobic, putative transmembrane regions (TMs) that are characteristic of GPCRs. As observed before with the partial cDNA fragment, the full-length receptor showed highest similarity to members of the SST/galanin/opioid family of GPCRs; with the exception of TM4 (<40% similarity) the amino acid similarities in the TMs ranged from 50 to 70% (Figure 1). The *Drosophila* orphan receptor exhibits several characteristic features of GPCRs such as N-linked glycosylation sites (Asn28, Asn36, Asn57), a potential palmitoylation site (Cys364) shortly after TM7, which may anchor part of the intracellu-

lar C-terminus to the lipid bilayer, and several potential sites for phosphorylation by protein kinase A (Arg-X-Ser/Thr) in the third intracellular loop (Thr290) and protein kinase C (Thr/Ser-X-Arg/Lys) in the intracellular (second, third) loops (Ser184, Thr187, Ser282) as well as within the intracellular C-terminal region (Thr379).

Northern blot analysis of poly(A)⁺ RNA from *Drosophila* heads hybridized with a DNA probe derived from the receptor-encoding cDNA clone revealed a single transcript of ~3.7 kb (Figure 2) indicating that the isolated cDNA most likely lacks ~1.8 kb at the 3' untranslated region. The developmental and tissue-specific expression pattern was studied by RT-PCR experiments on RNAs isolated from *Drosophila* embryos, pupae, larvae and adult animals as well as isolated heads and bodies. The receptor transcript is absent from embryos, but appears later in development in pupae and larvae and is present in heads and bodies of the adult animals (Figure 2), suggesting that as with the mammalian SST/galanin/opioid receptor family the expression of the orphan *Drosophila* receptor is not restricted to the central nervous system.

Comparison with the DDBJ/EMBL/GenBank database revealed that the cloned receptor is represented in a genomic clone (121E7) which has been localized to the X chromosome. At present the receptor has been mapped

Fig. 2. Expression of the novel *Drosophila* receptor. (A) Northern blot analysis. Poly(A)⁺ RNA (5 µg) from *Drosophila* heads was subjected to Northern blot analysis. Hybridization was performed with an α -³²P-labeled cDNA fragment covering the entire coding region of the *Drosophila* receptor. (B) RT-PCR analysis. PCR was performed on cDNA samples obtained from tissues and developmental stages of *D.melanogaster*. Primers were chosen to amplify the full coding region of the receptor or a 320 bp fragment of *D.melanogaster* actin. The respective sizes of the amplified fragments are indicated by bars. In all cases the integrity of the RNA preparation was ascertained by a control PCR with primers specific for *Drosophila* actin.

Fig. 3. Schematic organization of the orphan *Drosophila* receptor gene. Numbering of the receptor gene is obtained from genomic clone 121E7 (DDBJ/EMBL/GenBank database), that of the mRNA is as stated for the cDNA sequence; mRNA numbering indicates the position of exon-intron boundaries; solid black line of the gene, introns; solid grey line of mRNA, coding regions; thin grey line, 5'- and 3'-UTR; E, exon (grey boxes); TM, regions coding for the transmembrane domains (black boxes with white lettering).

roughly to 4F-5A, a region known for behavioral (flightless mutant *flt-b*; olfactory defective mutants *ota-2* and *ota-7*) and anatomical brain mutants (*no-bridge nob*; small mushroom bodies *smu*). The cDNA sequence reported here matches the genomic clone in 11 clearly identifiable exons (Figure 3). As noted in the *Drosophila* genome project for the genomic clone 121E7, a partial cDNA structure has been assigned, which is however incorrect as several exons were not accurately predicted, most likely reflecting the inaccuracy of the Genefinder software program used. This is not too surprising because the exon-intron boundaries in the novel *Drosophila* receptor gene are frequently located within the transmembrane encoding regions, quite in contrast to the organization of the respective mammalian genes, which are often intronless in their coding regions (Richter *et al.*, 1991).

Identification of the natural ligand by the 'reverse physiology' strategy

The reverse physiology approach is based on screening a functional expressed orphan receptor against known

bioactive compounds or, alternatively, against purified peptides isolated from the respective tissue. Reverse-phase HPLC in combination with mass spectrometry and peptide sequencing should eventually result in the identification of the bioactive ligand. Because of its structural similarities with members of the SST/galanin/opioid receptor family (Kreienkamp *et al.*, 1997), we assumed that the identified orphan *Drosophila* receptor might also couple to the Gi/Go class of G proteins. It has been shown previously that agonist binding to mammalian SST or opioid receptors leads to the activation of G-protein-gated inwardly rectifying potassium channels (GIRK) (Kreienkamp, 1999). Gating of these channels is due to a direct interaction with free $\beta\gamma$ subunits of heterotrimeric G proteins and can be measured in frog oocytes by whole-cell voltage-clamp recordings. Hence frog oocytes, co-expressing the *Drosophila* receptor and the mouse GIRK1, were exposed to various bioactive peptides and electrophysiologically recorded for inward potassium currents. However, none of the mammalian peptides tested, e.g. SST14, SST28, galanin, Leu- or Met-enkephalin, or the insect peptide

Fig. 4. Functional expression of the novel *Drosophila* receptor in frog oocytes. *Xenopus* oocytes were injected with cRNAs coding for the novel receptor and the GIRK1 potassium channel subunit, respectively (A and B) or the GIRK1 potassium channel alone (C). Measurements were initiated by changing from ND-96 to high-potassium media (hK, downward arrows; upward arrows, wash with ND-96). Solid bars, duration of agonist applications; in (A) agonists were applied in hK at a 1 μ M concentration. *Drosophila* head extracts were diluted with hK as indicated (B and C). Enk, enkephalin; SST, somatostatin.

proctolin were active in the frog oocyte expression system (Figure 4A). Alternatively, the orphan *Drosophila* receptor was expressed in the absence of GIRK subunits and measurements were performed in normal Ringers solution, thereby trying to activate the phospholipase C pathway known to generate calcium-induced chloride currents. Again no measurable response was detected (data not shown). In the absence of any functional response induced by mammalian neuropeptides, we searched for potential candidates in extracts from *Drosophila* heads. Indeed, crude *Drosophila* head extracts at a dilution of 1:7000 induced a strong GIRK-mediated inward potassium current in frog oocytes (Figure 4B), while no response was detected in control oocytes injected with GIRK cRNA only (Figure 4C). In addition, a response was also detected by using a similar extract from *Drosophila* bodies; however, the agonist appeared to be present at a lower concentration, as a dilution of 1:1000 had to be used to elicit a significant current (data not shown).

The crude extract from 10 g of *Drosophila* heads was purified by cation-exchange chromatography; fractions eliciting a significant inward potassium current in the frog oocyte expression system were combined and subjected to four consecutive purification steps by reverse-phase HPLC until an apparently homogeneous fraction could be obtained. Aliquots of the biologically active fraction were analysed by mass spectrometry; a single mass of 925 Da could be detected suggesting that the peptide had indeed been purified to homogeneity (Figure 5A–D). Sequence analysis was performed by post-source decay, and the combined data revealed the following sequence: NH₂-Ser-Arg-Pro-Tyr-Ser-Phe-Gly-Leu/Ile-amide (Figure 6). In the

last position leucine/isoleucine could not be differentiated because of their identical molecular weights.

As indicated in Table I, this peptide is structurally related to the AL peptide family and we therefore named the novel *Drosophila* receptor allatostatin receptor (AlstR). There is no similarity between AL and the mammalian peptides SST, galanin or enkephalins, which is in line with our functional expression data demonstrating that neither of the mammalian peptides activated the AlstR (Figure 4A). As all peptides of this group identified so far exhibit a C-terminal sequence consisting of Tyr-Xaa-Phe-Gly-Leu-amide, we inferred that the *Drosophila* AL should also terminate with a leucine-amide (Table I). To verify this the peptide Ser-Arg-Pro-Tyr-Ser-Phe-Gly-Leu-amide was synthesized and used as ligand in the frog oocyte expression system. The synthetic peptide activated the expressed *Drosophila* receptor efficiently and with a very high apparent affinity. In contrast, no response was obtained from control oocytes when the AlstR was replaced by the rat SSTR2 or when GIRK1 alone was assayed (Figure 7A). A dose-response curve obtained from AlstR-expressing oocytes reveals an EC₅₀ of 55 pM for the *Drosophila* AL peptide (Figure 7B). Several commercially available ALs from *Diploptera punctata* were also tested. Type A ALs III and IV from *Diploptera punctata* activated the *Drosophila* AlstR with similar high affinity, exhibiting EC₅₀ values of 147 and 156 pM, respectively. Type B AL, which contains a considerably longer N-terminus but also terminates in the typical last five amino acid residues, also activates the AlstR but does so with a 20-fold lower affinity (Figure 7B).

Discussion

The data reported here demonstrate that AL is the natural ligand for the identified *Drosophila* receptor, which shares structural and functional properties with the mammalian SST/galanin/opioid receptor family (Darlison *et al.*, 1997; Kovoor *et al.*, 1997; Kreienkamp, 1999); when co-expressed with GIRK in frog oocytes, the ALstR activated the inward potassium currents efficiently, whereas activation of phospholipase C followed by activation of calcium-induced chloride currents was not observed. Evidently the *Drosophila* AlstR couples to a G protein of the Gi/Go family; whether the receptor also couples negatively to the cyclic AMP second messenger pathway has to await further analysis.

The ligand was identified by a reverse physiology strategy (Meunier *et al.*, 1995; Reinscheid *et al.*, 1995; Tensen *et al.*, 1998), which makes use of the combined expression of the orphan receptor and GIRK in frog oocytes and their sensitive electrophysiological recording in combination with HPLC purification of the ligand and its mass spectrometric analysis. Thus, the AL peptide could be purified to homogeneity from <10 g of *Drosophila* tissue. The new *Drosophila* AL is structurally identical to the lepidopteran helicostatin 3 peptide (Duve *et al.*, 1997a) sharing the conserved C-terminal pentapeptide YXFGL-amide present in all the other members of the AL peptide family (Table I). The pentapeptide is required and sufficient for agonistic effects in biological assays, whereas the N-terminal part of the molecule may vary considerably in length and in sequence even within

Fig. 5. Purification of the natural agonist of the *Drosophila* receptor. (A) Cation-exchange chromatography. Separation was performed on a MonoS cation-exchange column. Peptides were eluted with a gradient from 10 mM to 1 M ammonium formate, pH 4.0, in 10% methanol. (B) Reverse-phase HPLC. The active fractions (for details, see Materials and methods) from the cation-exchange column were applied directly to a reverse-phase (C18) column and eluted with a linear gradient from 0 to 70% acetonitrile in 0.1% trifluoroacetic acid. (C) Final HPLC purification. After two more HPLC steps, final purification was achieved by reverse-phase HPLC on a C8 column. Active fractions in all experiments are indicated by solid bars. (D) MALDI mass spectrometry of the purified peptide. A single peak of relative mass 925.49 ($M+H$, monoisotopic mass) was detected, the two smaller ones to the right represent sodium and potassium adducts of this peak. a.i., absolute intensity; m, mass; z, charge.

the multiple copies of ALs that can be produced from one polypeptide precursor by proteolytic processing (Donly *et al.*, 1993; East *et al.*, 1996; Vanden Broeck *et al.*, 1996; Veenstra *et al.*, 1997).

AL was first identified in the cockroach *Diploptera punctata* as an inhibitor of juvenile hormone synthesis in the corpora allata (Woodhead *et al.*, 1989). It was later shown that ALs are present not only in various insects (Davis *et al.*, 1997) but also in other invertebrates including crabs and lobsters (Duve *et al.*, 1997b; Skiebe, 1999). Owing to their wide abundance, ALs have been shown to mediate multiple biological functions in insects. The original inhibition of juvenile hormone synthesis could not be reproduced in the dipteran *Calliphora* (Duve *et al.*, 1993). In *Drosophila*, immunological analysis revealed AL-like immunoreactivity in various cell populations, but not in those fibres that terminate on the corpora allata; instead, immunoreactivity was demonstrated in various ganglia in the central nervous system (Yoon and Stay, 1995), consistent with our finding that both the receptor and its agonist are strongly expressed in heads. The peptide has especially been found in interneurons (Yoon and Stay, 1995), which is a feature shared by the mammalian peptides that activate the structurally related SSTRs (Somogyi *et al.*, 1984). This analogy may reflect a general importance of inhibitory neuropeptides in interneurons. In addition, AL has been shown to inhibit the movement of visceral muscles in the gut, and AL has therefore been

termed a brain–gut peptide (Gäde *et al.*, 1997). It appears possible that these effects are also mediated by the AlstR reported here, as it is also strongly expressed in the bodies of adult flies.

The *Drosophila* AlstR is structurally related to the mammalian neuropeptide receptors. Figure 8 shows that the compared receptors fall into three groups. The AlstR is most closely related to the galanin receptors, followed by the five SSTR subtypes, whereas the third group is formed by the opioid receptors. Because of the low number of known insect GPCRs, any phylogenetic relationships with the *Drosophila* AlstR are of a preliminary nature. The listed tachykinin and neuropeptide Y receptors from *Drosophila* are only distantly related to the AlstR and most likely are evolutionarily as far apart as their vertebrate counterparts from the mammalian galanin/SSTR/opioid receptor family. The structural relationship of the novel AlstR to the mammalian counterparts with apparently completely unrelated ligands is mainly restricted to those core regions of the seven TMs facing the intracellular side of the membrane known to interact with G proteins. In contrast, the sites determining the specificity of ligand binding in the third extracellular loop and the neighbouring region of the sixth TM of the mammalian galanin/SSTR/opioid receptor family are clearly distinct from those of the AlstR (Figure 1). This may suggest that during evolution the basic scaffold of this family of receptors has been conserved with respect to its G protein-binding

Fig. 6. Peptide sequence analysis. Portions of MALDI-PSD time-of-flight spectrum showing all peaks that were relevant for the establishment of the amino acid sequence of the purified peptide. The amino acid sequences of the peaks given in single letter code are the results of the assignment procedure as described by Chaurand *et al.* (1999); masses are not shown. A tyrosine residue was identified as a constituent of this peptide by the appearance of a characteristic immonium ion (left). For sequence analysis, the b- and y-fragment series (corresponding to cleavage at the peptide bond and yielding N- and C-terminal fragments, respectively) derived from the full sequence could be partially established. Sequence analysis was facilitated by the observation that one proline was present, which usually serves as major breaking point; thus a new series of b-fragments was observed with proline as the first amino acid. The loss of 17 mass units corresponds to the loss of ammonia, indicative of lysine or arginine in a fragment. The loss of 18 mass units corresponds to the loss of water, which may indicate the presence of serine or threonine. The loss of 28 mass units indicates the presence of a fragment of the a-series, where cleavage in the peptide chain did not occur between the C- and the N-atoms of the peptide bond but between the α -C-atom and the carbonyl-C-atom, leading to the loss of a carbonyl group ($m = 28$) from the N-terminal fragment (Chaurand *et al.*, 1999). m, mass; z, charge.

Table 1. Comparison of the novel AL-like peptide from *Drosophila* with AL peptides from Lepidoptera and *Diptera*

Peptide	Sequence
AL-like peptide (<i>Drosophila melanogaster</i>)	SRPYSFGL-NH ₂
Helicostatin 3 (lepidopteran)	SRPYSFGL-NH ₂
AL I (<i>Diptera punctata</i>)	APSGAQRLYGFGL-NH ₂
AL III (<i>Diptera punctata</i>)	GGSLYSFGL-NH ₂
AL IV (<i>Diptera punctata</i>)	DRLYSFGL-NH ₂
AL type B (<i>Diptera punctata</i>)	AYSIVSEYKRLPVYSFGL-NH ₂
SST 14 (ovine)	AGCKNFFWKTFTSC-OH
Leu-enkephalin (bovine)	YGGFL-OH
Galanin (human)	GWTLNSAGYLLGPHAVGNHRS-FSDKNGLTS-OH

Note there is no homology with the mammalian peptides SST, galanin or enkephalin. Residues identical in the AL peptide family are indicated by bold letters.

site, which is supported by our functional expression studies that both AlstR and the SST/opioid receptors activate the same mammalian GIRK channel. The receptor domains conferring ligand specificity may have evolved rapidly to accommodate new peptide ligands.

It remains to be seen whether there are more receptor subtypes of the AlstR, as is known for the mammalian SST/galanin/opioid receptor family. There is circumstantial evidence that at least two different AL-binding sites exist in cockroaches (Cusson *et al.*, 1991; Yu *et al.*, 1995). Using the receptor sequence described here, other related

subtypes will certainly be discovered through either homology screening or the efforts of the *Drosophila* genome project.

Materials and methods

Preparation of RNA from *Drosophila* tissues

Embryos were obtained by culturing flies overnight on apple juice–agar plates. The next day, the embryos were collected by rinsing the plates with water and collecting on an analysis sieve (60 μ m; Neo-Lab, Heidelberg, Germany). Larvae and pupae were collected using the same method. Adult flies were treated with liquid nitrogen and then frozen overnight at -80°C . The next day, the heads were separated from the bodies with the help of analysis sieves (150 μ m, 710 μ m, 1000 μ m; Retsch, Haan, Germany). All tissues were collected into lysis/binding buffer described by the Dynabead protocol and homogenized using an Ultra Turrax. Poly(A)⁺ RNA was isolated using Dynabeads oligo(dT)25 (DynaL, Hamburg, Germany) according to the manufacturer's instructions.

RT-PCR

For reverse transcription, poly(A)⁺ RNA (~1 μ g) was incubated for 5 min in a 70°C water bath. First-strand cDNA was synthesized in a final volume of 50 μ l, using 4 μ g oligo p(dT) and reverse transcriptase (MBI Fermentas, Vilnius, Lithuania) at 37°C for 90 min. Control reactions, from which reverse transcriptase was omitted, were set up in parallel. A total of 5 μ l of each reaction was then amplified using the degenerate oligonucleotide primers 5'-GCGGAATTC(C/T)(T/A)(C/T)TGGCCCTT(C/T)GG-3' and 5'-GACGGATCC(G/A)AAXGGXA(G/A/T)CCA(G/A)CA-3'. These recognize the nucleotide sequences that encode amino acid residues (H/Y)WPF (positions 122–125 in rSSTR1) and W(M/L)PF (positions 285–288 in rSSTR1; Meyerhof *et al.*, 1991), respectively, which are common to mammalian SSTRs. PCR products of the expected size (500–700 bp) were isolated, cloned into pcDNA3

Fig. 7. Activation of the *Drosophila* ALstR by the synthetic *Drosophila* AL peptide. (A) Oocytes expressing the ALstR and GIRK1 (left) or GIRK1 alone (upper right) or GIRK1 and rat SSTR2 (lower right) were stimulated with 10 nM of the synthetic *Drosophila* AL peptide in hK media (solid bars). Note the absence of a response in oocytes expressing GIRK1 alone or in combination with rat SSTR2. (B) Dose-response curve for the synthetic *Drosophila* AL and *Diploptera* ALs. Agonist-activated GIRK currents were measured as described in Figure 4; peak currents were normalized against the maximum currents obtained for each oocyte. The dose-response data were then subjected to non-linear regression analysis using GraphPad Prism software (GraphPad Inc., San Diego, CA). EC₅₀ values were 55 pM for the *Drosophila* AL peptide (squares), 147 pM for AL IV (triangles), 156 pM for AL III (circles) and 3.3 nM for AL type B (diamonds).

vector taking advantage of restriction endonuclease recognition sites incorporated into the 5' ends of the PCR primers, and sequenced.

To obtain a full-length cDNA, the RACE technique was applied using the first-strand cDNA derived from *Drosophila* head RNA. Primers for 3'RACE were 5'-GCTGCAAACCTTCCGCAGAGTC-3' and 5'-AGG-GAATTCGGCGCGTCACCCGAATGGTTG-3'. Primers for 5'RACE were 5'-CAGGAAGCGATCAAAGGACATCAG-3' and 5'-CACGGA-TCCACAATCATGTACTGGACAAAC-3'. RACE reaction products were cloned into pBluescript and sequenced. Based on this sequence information, primers were designed recognizing the sequence surrounding the ATG start codon (5'-GCCAAGCTTATGGCTGGCCATCAGTC-GC-3') and the stop codon (5'-CCTCTAGATTAGCATTTCATA-TTGGACC-3') of the novel receptor cDNA and used to amplify a full-length cDNA from *Drosophila* heads. The PCR product of 1182 bp was cloned into pcDNA3, and several independent clones were sequenced in their entire length in order to verify the correctness of the cDNA sequence.

Northern blot analysis

Poly(A)⁺ RNA (5 µg) was separated on 1% agarose gels in 40 mM morpholinopropane sulfonic acid, 10 mM sodium acetate, 6.6% formaldehyde. After electrophoresis, RNA was transferred to nylon filters (Hybond N, Amersham, Braunschweig, Germany) and UV crosslinked to the membrane. Filters were hybridized at 50°C in 5× SSC, 10× Denhardt's solution, 100 µg/ml denatured salmon sperm DNA, 0.1% SDS, 50%

Fig. 8. Sequence relationship of the insect AL, tachykinin and neuropeptide Y receptors and rat receptors for SST, galanin and opioids depicted in the form of a dendrogram using the PILEUP algorithm [Genetics Computer Group (GCG), Madison, WI]. The lengths of the horizontal lines indicate reciprocally the sequence similarities. The bar at the bottom corresponds to a mutation rate of 10 substitutions per 100 residues; evolutionary distances were calculated using the Jukes-Cantor method in the Distances program of the GCG package; the phylogenetic tree was reconstructed with the Growtree program (also from the GCG package); here the unweighted group pair method using arithmetic averages was employed. Polypeptide sequences were obtained from the ExPASy Molecular Biology Server of the Swiss Institute of Bioinformatics. dros, *Drosophila*; TKR, tachykinin-like peptides receptor 2; NK, neurokinin receptor (also known as tachykinin-like peptides receptor 1); NPY, neuropeptide Y receptor; OFQ, orphanin FQ receptor.

formamide for 16 h. Blots were washed twice in 2× SSC/0.1% SDS for 5 min at room temperature and twice at 55°C for 15 min. Exposure of the filters to X-ray films was for 96 h at -80°C.

Expression in *Xenopus* oocytes

For functional expression in frog oocytes, the receptor cDNA was subcloned into the *Xenopus* expression vector pGEMHE which contains *Xenopus* globin 5'- and 3'-untranslated regions. The plasmids were linearized with *Pst*I, and RNA was transcribed *in vitro* using T7 RNA polymerase (MBI Fermentas, Vilnius, Lithuania). Co-expression with the GIRK1 cRNA in frog oocytes was performed as described previously (Kreienkamp *et al.*, 1997). For recording, oocytes were superfused with ND-96 medium (96 mM NaCl, 2 mM KCl, 2.5 mM CaCl₂, 1 mM MgSO₄, 5 mM HEPES pH 7.5) and clamped at -80 mV. For measurements of agonists, the medium was changed to high K⁺-medium (hK; ND-96 with 96 mM KCl and 2 mM NaCl); after the initial inward current reached a plateau, agonists were applied in the same medium. Agonist treatment was terminated by washout with hK and subsequent switching to ND-96 media.

Extraction of *Drosophila* heads

For purification of the native ligand of the cloned receptor, a supernatant fraction from *Drosophila* heads was prepared by grinding heads in liquid nitrogen; the ground material was homogenized in 10 mM Tris-HCl buffer pH 7.5, 280 mM sucrose, 0.01% w/v Na₃N, 0.1 mM phenylmethylsulfonyl fluoride and centrifuged (Schloss *et al.*, 1988). Material corresponding to ~10 g of heads was adjusted to 0.5 M acetic acid and 10 mM ascorbic acid and boiled for 10 min. Precipitated proteins were removed by centrifugation, the supernatant fraction applied to C18-SepPak cartridges (Waters, Eschborn, Germany), washed with 5 ml of 0.1% trifluoroacetic acid (TFA), and eluted with 80% methanol, 0.1% TFA. After evaporation and lyophilization, the residual peptide mixture was dissolved in 10 mM ammonium formate (pH 4.0) and applied to a MonoS cation-exchange column (Pharmacia, Freiburg, Germany) linked to an LKB HPLC system. Peptides were eluted at a flow rate of 1.0 ml/min with a linear gradient from 10 mM ammonium formate to 1 M ammonium formate (pH 4.0) in 10% methanol. Aliquots

of fractions were lyophilized twice in 1 ml of water and dissolved in hK buffer for activity measurements. Active fractions were separated further on a C18 reverse-phase HPLC column, followed by a C4 column and two runs on a C8 column.

Peptide analysis

The HPLC-purified peptide was analysed by mass spectrometry using a Bruker Reflex mass spectrometer (matrix-assisted laser desorption mass spectrometry). The sequence was determined by analysis of fragment ions generated by post-source decay (Chaurand *et al.*, 1999), using the FAST™ method. A matrix-assisted laser desorption ionization-post-source decay (MALDI-PSD) time-of-flight spectrum was recorded using α -cyano-4-hydroxy cinnamic acid as a matrix; acquisition was at 27.5 kV under continuous extraction conditions; reflector voltage was stepped from 30 to 1.27 kV, and the spectrum was constructed using the FAST method from Bruker-Daltonics GmbH (Bremen, Germany).

To verify that the purified molecule is indeed a receptor ligand, the peptide was custom-made by Genemed Synthesis Inc. (San Francisco, CA). All other peptides were obtained from Bachem (Hannover, Germany).

Accession number

The sequence data for the *Drosophila* seven transmembrane receptor have been submitted to the DDBJ/EMBL/GenBank database under accession No. AF163775.

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