Functional Annotation of Two Orphan G-protein-coupled Receptors, Drostar\textsuperscript{1} and -2, from Drosophila melanogaster and Their Ligands by Reverse Pharmacology*\textsuperscript{1}

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By combining a Drosophila genome data base search and reverse transcriptase-PCR-based cDNA isolation, two G-protein-coupled receptors were cloned, which are the closest known invertebrate homologs of the mammalian opioid/somatostatin receptors. However, when functionally expressed in Xenopus oocytes by injection of Drosophila orphan receptor RNAs together with a coexpressed potassium channel, neither receptor was activated by known mammalian agonists. By applying a reverse pharmacological approach, the physiological ligands were isolated from peptide extracts from adult flies and larvae. Edman sequencing and mass spectrometry of the purified ligands revealed two decapentapeptides, which differ only by an N-terminal pyroglutamate/glutamine. The peptides align to a hormone precursor sequence of the Drosophila genome data base and are almost identical to allatostatin C from Manduca sexta. Both receptors were activated by the synthetic peptides irrespective of the N-terminal modification. Site-directed mutagenesis of a residue in transmembrane region 3 and the loop between transmembrane regions 6 and 7 affect ligand binding, as previously described for somatostatin receptors. The two receptor genes each containing three exons and transcribed in opposite directions are separated by 80 kb with no other genes predicted between. Localization of receptor transcripts identifies a role of the new transmitter system in visual information processing as well as endocrine regulation.

Insect development and behavior are largely controlled by hormones and neurotransmitters often identified using a diverse array of bioassays. Besides the biogenic amines and the steroid-like hormones, insect hormones have been frequently classified as neuropeptides, which are widely distributed throughout the invertebrate kingdom (1, 2). Despite the large number of neuropeptides, the number of known cognate receptors in insects is still rather limited, with only a few examples in Drosophila that have been cloned based on homology with mammalian G-protein-coupled receptors (GPCRs)\textsuperscript{1} (i.e. neuropeptide Y-like and tachykinin-like receptors) (3, 4). With the completion of the Drosophila genome project, a more thorough analysis of neuropeptide/receptor relations in insects is now possible. Whereas this genome data base allows the identification of peptide hormones previously isolated from other insect species as part of larger precursors (5), Drosophila GPCR-like sequences have been predicted mostly based on structural analogy of the transmembrane regions to mammalian neuropeptide receptor groups (6).

Structural evidence for the existence of ligands identical or similar to their mammalian neuropeptide counterparts are lacking when searching the Drosophila genome data base. This may indicate that in insects these receptors are activated by an entirely different set of ligands. This view is supported by data reported here on the identification of two novel GPCRs from Drosophila melanogaster, termed Drostar1 and -2, which are structurally related to the mammalian opioid/somatostatin receptor family yet are activated by peptide ligands, pyro-Glu and Glu-allatostatin C, unrelated to any known mammalian agonists.

MATERIALS AND METHODS

Molecular Cloning—Several PCR primers were designed to amplify SST-like receptor cDNAs from Drosophila RNA (7); 5'-attgcatatggaaggcsgttacagggg-3' and 5'-taagttttcaaaatgctgtgtg-3' were used to amplify a 1.4-kb DNA corresponding to the CG7285 gene product of the Drosophila genome data base. 5'-gtctgcatatggaccttta-3' and 5'-ttttcattagttactggacagc-3' were used to amplify a central fragment of the CG13702 gene product. Based on the putative 3'-end of the coding region of this gene (predicted by similarity to the CG7285 gene product), we used primers 5'-tgctgcatatggacctttg-3' and 5'-gtctgcatatggacctttg-3' to amplify the 3'-end of this cDNA. The 5'-end of this cDNA was obtained by 5'-rapid amplification of cDNA ends (7). All PCR fragments were cloned into TOPO vectors (Invitrogen) and sequenced. Site-directed mutagenesis was performed by PCR using mutagenic primers and Vent DNA polymerase.

Functional Expression in Xenopus Oocytes—Wild type and mutant receptor cDNAs were cloned into a modified pGEMHE expression vector, which carries a sequence coding for the signal peptide of the 5-hydroxytryptamine receptor 3 (amino acid sequence MVLWLQAL-LALLLPTSLAQGEVDI); the pGEMHE vector itself contains 5'- and 3'-untranslated regions derived from the Xenopus laevis glolin gene,
which stabilize exogenous RNA in *Xenopus* oocytes (8). After linearization of the vector with NheI, cRNA was transcribed with T7 RNA polymerase and co-injected with the mouse GIRQ1 cRNA into *Xenopus* oocytes (7). Agonist-induced potassium currents were recorded in high potassium (hK) solution under whole cell voltage clamp conditions (7). For the testing of fractions obtained by various chromatographic steps, aliquots were lyophilized and dissolved in hK buffer.

**Peptide Purification**—Crude peptide extracts from adult flies and a mixture of larvae and pupae (both about 50 ml of material) were prepared by homogenizing frozen samples in 200 ml of 0.5M acetic acid, a crude purification of this extract was achieved by gel filtration using a Sephadex G-25 column (43 × 1.4 cm; in 0.1 M acetic acid); active fractions from both the adult flies and the larvae/pupae mixture were pooled and further purified using Vydac C-8, C-18, C-4, and Nucleosil C-8 (two times) reverse phase HPLC columns.

**Sequence by Edman Degradation and Mass Spectrometric Analysis**—Peptide sequences were determined by standard Edman degradation using an automatic pulsed-liquid protein sequencer (476A; Applied Biosystems, Foster City, CA). Mass spectrometric analysis was performed with a hybrid tandem mass spectrometer (QTOF II; Micromass, Manchester, UK) equipped with a nanoelectrospray ion source. Samples were purified by binding to C18 reverse phase material in a pipette tip (ZipTip; Millipore Corp.) and eluted with 1 l of 50% methanol, 5% formic acid into a gold-coated borosilicate vial for nanoelectrospray measurements.

**Synthetic Peptides**—Synthetic, linear Gln-allatostatin C (QVVRQRCYPHFPSRC) was obtained by custom synthesis from Genemed Synthesis, Inc. (South San Francisco, CA). For oxidation, the peptide was treated with K2Fe(CN)6 (9) for reduction with 1 min dithiothreitol (30 min, 65 °C). Conversion of linear Gln-allatostatin C peptide into pyro-Glu-allatostatin C was achieved with glacial acetic acid at 95 °C for 60 min, followed by K2Fe(CN)6 oxidation as above. The purity and the identity of active agonists was then determined by reverse phase HPLC as well as by mass spectrometry.

**In Situ Hybridization and Immunocytochemistry**—Heads of adult flies were snap frozen in Tissue Tek (Sakura, Tokyo, Japan). Sections (10 μm) were dried for 1 h at room temperature and postfixed in 4% paraformaldehyde/phosphate-buffered saline, pH 7.4. Tissue was perfused with phosphate-buffered saline, pH 7.4, 0.3% Triton X-100. Prehybridization was done with Ultrahyb (Ambion, Austin, TX) supplemented with salmon sperm and tRNA for 2 h at 60 °C. cRNA was in vitro transcribed (Ambion) with digoxigenin UTP. Hybridization was carried out overnight at 60 °C in prehybridization buffer followed by immunohistochemical detection with alkaline phosphatase-labeled anti-galanzin antibody (Roche Molecular Biochemicals).

Paraformaldehyde-fixed and frozen sections from last instar larvae were processed for immunocytochemistry (10). *Manduca sexta* allatostatin C antibody (primary antibody) was diluted 1000-fold, and secondary antibody (Cy3-labeled goat anti-rabbit, Dianova, Hamburg, Germany) was diluted 200-fold. Fluorescence was visualized using a Leica DM-IRBE microscope equipped for epifluorescence.

**RESULTS**

**Receptor Identification**—When screening the *Drosophila* genome data base with mammalian somatostatin or opioid receptors as probes, two highly similar sequences partially overlapping with the predicted CG7285 and CG13702 gene products were detected on a contig (accession number AE003520) derived from chromosome 3L.

Using total RNA isolated from adult flies and primers based on conserved regions of the mammalian SSTR2 and on the coding sequences predicted from the *Drosophila* genome project annotation data base, we isolated and sequenced cDNAs coding for both receptors by reverse transcriptase-PCR. The CG7285 cDNA could be amplified using forward and reverse primers directed at the 5'- and 3'-ends of the predicted coding sequences; this was not possible for the CG13702 cDNA, since both ends of the coding region were incorrectly predicted. Here primers for the 3'-end were designed based on similarity to the CG7285 gene product, whereas the 5'-end of the cDNA was determined by 5'-rapid amplification of cDNA ends. Open reading frames of 1401 bp (CG7285) and 1467 bp (CG13702) were detected, coding for proteins of 467 and 489 amino acids, respectively (Fig. 1). The deduced protein sequences exhibit the characteristic pattern of GPCRs (seven TMs; N-linked glycosylation sites at the N terminus). Both receptors share 60% identical amino acid residues; considering only the TMs, this value increases to 76%, strongly suggesting that the two receptors are distinct members of a novel GPCR subfamily. When comparing both sequences with GPCRs present in the GenBank™ data base, mammalian SSTR2 appears as the closest relative (42% amino acid identity in the TMs), followed by the other SSTR subtypes and then the opioid receptors (Fig. 2). Other insect receptors (AlstR1 and -2) appear closer to the mammalian galanin receptor family.

**Functional Expression of the Two Receptors and Identification of Their Cognate Ligands**—Functional expression in *Xenopus* oocytes was achieved by coinjection of CG7285 cRNA together with GIRQ1 cRNA. Significant signals were recorded when oocytes expressing the novel *Drosophila* receptor were exposed to a crude peptide extract from *Drosophila* larvae or adult flies (Fig. 3A). Since weaker signals were also recorded from oocytes expressing the GIRQ1 channel alone, the specificity of signals could only be ascertained after Sephadex G-25 gel filtration of the crude peptide extracts (Fig. 3B). Signals could be detected in fractions corresponding to a molecular mass of
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Active fractions were further purified by reverse phase HPLC (Vydac C-8 column). The activity was eluted as two distinct peaks (Fig. 4A), which were purified separately. After three further reverse phase HPLC steps, the first (slightly more hydrophilic) active ligand was finally isolated in an apparently pure peptide fraction, as judged by UV absorbance of the HPLC elution profile (Fig. 4B). Analyzed by Edman degradation, this peptide revealed the sequence XVRYRQXYFNPLX. Table I shows that this sequence aligns with part of a hormone precursor sequence derived from the Drosophila genome data base and referred to as Drostatin precursor (11). The resulting decapentapeptide is reminiscent to allatostatin C isolated from the tobacco hornworm, M. sexta (12), except that in Manduca the first amino acid residue is a pyroglutamate, and in position 4 the tyrosine is replaced by phenylalanine (Table I). Our ability to sequence the Drosophila agonist of peak 1 (Fig. 4B) by Edman degradation indicated that the pyro-Glu modification was not present in the purified peptide. In agreement with this, mass spectroscopy of the pure peptide yielded a peptide mass of 1920.67, which corresponds to the predicted peptide mass of 1903.26 and the pyro-Glu peptide version elutes about 2 min later from a reverse phase Vydac C-8 HPLC column (data not shown), confirming the sequence alignment with the allatostatin C sequence.

The second peptide peak (Fig. 4) was purified accordingly, but due to its rather low abundance when compared with peak 1 it was analyzed by mass spectroscopy; the detected peptide mass of 1903.26 corresponded to the decapentapeptide carrying an N-terminal pyroglutamate, indicating that Drosophila contains two types of allatostatin C carrying N-terminally either a glutamate or a pyroglutamate; they are present in the peptide extract with an estimated ratio (in terms of agonistic activity) of 1:2 (pyro-Glu-allatostatin C/Gln-allatostatin C; see Fig. 4A). Accordingly, the two receptors were termed Drostar1 and -2 (for Drosophila allatostatin C receptors 1 and 2).

To verify our findings that the deduced sequences of the decapentapeptides are indeed that of the agonists of Drostar1 and -2, the linear Gln-allatostatin C peptide was custom-synthesized and tested for ligand activity. The linear peptide as obtained after synthesis yielded a rather low receptor-induced potassium current in the Xenopus oocyte system. However, after oxidation of the two cysteine residues (positions 7 and 14), strong agonist-induced currents were obtained, which were specific to oocytes expressing either Drostar1 or -2 and Drostar1 but which were not observed with oocytes expressing either GIRK1 alone or SSTR2/GIRK1 (Fig. 5, A–C). Strong currents were also induced by the peptide after generation of the N-terminal pyro-Glu modification, followed again by peptide oxidation. A comparison of HPLC elution profiles of synthetic, oxidized peptide carrying N-terminally either the pyro-Glu or Gln showed that the pyro-Glu peptide version elutes about 2 min later from a reverse phase Vydac C-8 HPLC column (data not shown), confirming that the second peak of agonistic activity obtained during the purification procedure (Fig. 4B) is the Drosophila pyro-Glu-allatostatin C reminiscent of the M. sexta peptide.

The reduced form of the Gln-allatostatin C peptide exhibited a potency that was about 100-fold lower than that of the oxidized form. To test whether the two Drostars discriminate between Gln- and pyro-Glu-allatostatin C peptides, dose-response curves were recorded. Both peptides were almost equally active at both receptors with EC_{50} values for GIRK1 activation in the low nanomolar range (Gln-allatostatin C: 9.45 nM at Drostar1, 7.0 nM at Drostar2; pyro-Glu-allatostatin C: 25.4 nM at Dro-
that the capacity to produce pyro-Glu-allatostatin C is re-
tinct in allatostatin is converted into both types of allastatin C. The reason
sequence is always a glutamine (Table I
the corresponding amino acid in the respective precursor se-
As known from other peptides starting with a pyroglutamate,
significantly to the ligand binding affinity toward its receptors.

fication at the N terminus of allatostatin C does not contribute
unidentified residues; *, stop codon.

TABLE I

Identification of the native agonists of the Drosophila receptors
A, alignment of the partial amino acid sequence of peak 1 (Fig. 4B)
by Edman degradation with the Drosophila precursor Drosta-
tin (DAP C103-121) sequence (11). Identical amino acid residues are
inverted. The sequence within DAP C flanked by basic amino acids
(underlined) predicts that this peptide is proteolytically released from
its precursor. B, sequence and mass spectrometric comparison of pep-
tide peak 1 or 2 (Fig. 4B) with Manduca allatostatin C (pyro-Glu-Alc)
(11). A comparison of mass spectroscopic data with the calculated val-
tues for Drosophila allatostatin C is given in the two right columns. X,
unidentified residues; *, stop codon.

| A. DAP-C | SKRQ | CVRYPQFNPICSP | XFPNKSCRPK 

| B. sequence | calc. mass | detected |
|-------------|------------|-----------|
| Glu-Alc | CVRYPQFNPICSP | 1920.89 | 1920.67 |
| pGlu-Alc | CVRYPQFNPICSP | 1902.90 | 1903.26 |
| pGlu-Alc | CVRYPQFNPICSP | 1886.88 |
| pGlu-Alc | CVRYPQFNPICSP | 1902.90 | 1903.26 |
| pGlu-Alc | CVRYPQFNPICSP | 1886.88 |

star1, 8.7 nM at Drostar2; Fig. 5B), suggesting that the modi-
fication at the N terminus of allatostatin C does not contribute
significantly to the ligand binding affinity toward its receptors.
As known from other peptides starting with a pyroglutamate,
the corresponding amino acid in the respective precursor se-
sequence is always a glutamine (Table I), suggesting that Dro-
statin is converted into both types of allatostatin C. The reason
for the presence of more unprotected versus protected allatostati-
C in Drosophila is at present not quite clear. It may reflect
that the capacity to produce pyro-Glu-allatostatin C is re-
stricted to a limited number of cells.

Mutational Analysis—The affinity and ligand specificity of
mammalian somatostatin receptors are determined by residues
in transmembrane region 3 (TM3; D124 in SSTR3 (13)) and in
those portions of TM6 and -7 facing the extracellular side of the
plasma membrane (14). These residues in Drostar1 were changed into the corresponding residues of the SSTR2 se-
quence by site-directed mutagenesis, and recombinant recep-
tors were challenged with pyro-Glu-allatostatin C and soma-
tostatin 14. A point mutation in TM3 reduced the affinity of
Drostar1 about 3-fold (Fig. 6), whereas a Drostar1 mutant
carrying SSTR2 sequence from the middle of TM6 to the middle
of TM7 was not activated by allatostatin C at all concentrations
tested. Neither mutant nor wild type receptors were activated
by SST14 (data not shown). It should be noted that when
constructs were transfected into human embryonic kidney
cells, there were no differences in the localization pattern be-
tween wild type and mutant receptors, indicating that the
transport to the cell surface was not impaired by the mutations
(data not shown).

Gene Organization and Expression Pattern—Both Drostars
are encoded by distinct genes located very close to each other at
a distance of about 80 kb with no other genes predicted between
them (Fig. 7). The open reading frames of both genes are
directed in opposite directions; both receptor mRNAs are tran-
scribed from three exons, including a small exon with 48 bp in
size that codes for a part of the sixth transmembrane region
(Fig. 1). Exon/intron boundaries, as deduced by comparison
with the genomic sequence, are entirely conserved between
both receptors. Although receptor-coding transcripts were pre-
dicted for both the 138-kb region (CG13702) and the 224-kb
region (CG7285) of this part of the Drosophila genome by the
GADFLY data base, predictions for both genes proved to be
inaccurate, since the small exon was apparently missed in each
case by the prediction software used; in the case of Drostar2,
both the N- and C-terminal part were also not correctly as-
signed (see data base entry, accession number AE003520).

In situ hybridization experiments revealed expression of
both Drostar genes within the optic lobes of adult flies, an area
devoted to the processing of visual information (Fig. 8A). In
the lamina region, these cells might represent lamina monopolar
cells, the main cellular components of the neuropil (15). In
addition, a region in the anterior median part of the brain, the
pars intercerebralis, has also few Drostar-expressing cells.
Since allatostatin C is also present in the pars intercerebralis
(16), the Drostar receptors on these cells might be involved in
autoregulatory processes. In addition, a group of cells at the
dorsal margin of the optic lobes also displayed allatostatin
C-like immunoreactivity (16), which may project to Drostar-
expressing cells of the optic lobe. The presence of both the
peptide and the receptor in the optic lobes suggests a function
in the modulation of visual information processing. Interest-
ingly, the disperse labeling within the neuropil regions indi-
cates Drostar expression in glial cells (Fig. 8A, arrowheads).

In larvae, Drostar expression was detected only in few cells
of the nervous system (Fig. 8B). In contrast, immunolabeling
of the peptide was found in the brain and corpora allata of the
larvae, presumably on axon terminals arising from the pars
intercerebralis of the brain (Fig. 8C). The allatostatin C peptide
may be released from these terminals into the hemolymph or
onto the corpora allata; thus, allatostatin C might function as
the main allatostatic activity in Drosophila. In agreement,
injection of pyro-Glu allatostatin C into the hemolymph of
second instar larvae led to a slightly shortened time until they
undergo pupariation (data not shown).

DISCUSSION

The data presented here identify two novel receptors, Dro-
star1 and -2, from the fruit fly and their cognate ligands as
pyro-Glu-allatostatin C and Gln-allatostatin C. Different peptides have been described as allatostatins based on their ability to inhibit juvenile hormone synthesis from the retrocerebral corpora allata complex of insects. They can be grouped into the allatostatin subfamilies A, B, and C. Members of subfamily A share the conserved C-terminal peptide motif YXFGL-amide, whereas their N terminus may vary in sequence and length. As reported, the conserved pentapeptide is sufficient to activate the cognate invertebrate AlstR1 and -2 receptors (7, 17). Members of the allatostatin B peptide family are characterized by the C-terminal motif XWXXXXXXW-amide. Neither of the two sequence motifs are present in the allatostatin C peptides of Manduca sexta or Drosophila. Thus, only the allatostatin C peptides activate the two Drostars, whereas no cross-activation of the two receptors occurs when complemented with allatostatin A peptides (Table II); conversely, AlstR1 is not activated by allatostatin C. As in M. sexta, our immunocytochemical data show that in Drosophila allatostatin C is present in the corpora allata presumably on axon terminals arising from the pars intercerebralis of the brain (Fig. 8), pointing to an allatostatic function. Using antibodies against an allatostin A-type peptide allatostatin-like immunoreactivity in Drosophila has been reported earlier mainly in nerve cells but not in fibers terminating on the corpora allata (18), also suggesting that in Drosophila allatostatin C rather than an allatostatin A is involved in the regulation of juvenile hormone synthesis. This assumption is supported by the observation that second instar larvae injected with allatostatin C show a slightly shortened period until they start pupariation. On the other hand, pyro-Glu allatostatin C does not reduce neurotransmitter release at the.

Fig. 5. Functional analysis of synthetic allatostatin C peptides. Synthetic untreated Gln-allatostatin C (untt. Gln-AlC) was chemically modified to yield oxidized (Gln-AlC; reduced (red. Gln-AlC), or pGlu-AlC (oxidized). Peptides were applied at a concentration of 1 μM to oocytes coexpressing Drostar1 and GIRK1 (A), GIRK1 alone (B), or rat SSRT2 and GIRK1 (C). In the SSRT2 experiment, 1 μM SST-14 was used as a positive control. The bars indicate the time of application of the respective peptides. Downward arrows, hK medium; upward arrows, ND-96 medium. D, dose-response relationship for the activation of Drostar1 (left) and Drostar2 (right) by synthetic allatostatin C peptides. Drostar1 or -2 was coexpressed with GIRK1 in Xenopus oocytes; agonists (squares, Gln-allatostatin C; triangles, pGlu-allatostatin C) were applied in hK medium; peak currents recorded from 4–6 oocytes at different agonist concentrations are depicted as the fraction of the maximum response obtained for each oocyte. Curves were obtained by fitting the data using nonlinear regression analysis in the GraphPad Prism software (GraphPad, San Diego, CA).

Fig. 6. Functional analysis of mutant Drostars. Drostar1 cDNA was mutagenized to yield the T140D mutant (in TM3) and a chimera where residues Val286–Asn329 of Drostar1 were replaced with the homologous Val264–Asn304 segment of human SSRT2 (GenBank accession number M81830). Mutant and wild type receptors were expressed as in Fig. 5, and dose-response curves were generated using pGlu-allatostatin C, as in Fig. 5. Squares, wild type receptor; triangles, T140D mutant; circles, Drostar/hSSRT2 chimera.

Fig. 7. Localization of Drostar1 and -2 on chromosome 3 (left arm). Numbers refer to positions in the contig described by the Drosophila genome project; arrows indicate the direction of transcription for the two genes. No potential gene is predicted between 138 and 223 kb on this contig. Large letters, exons 1–3 (E1–3); small letters, introns (boundaries underlined); gray sections, small exon 2 of Drostar1 and -2.

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were detected in few cell bodies in the central nervous system (arrowheads in the optic lobe (lam), section of a larvae. Immunostaining of cells in brain (heads and corpora allata (ca)). Specific hybridization was detected in the optic lobe (ol) and the pars intercerebralis (pi). At higher magnification of optic lobe, numerous labeled laminar monopolar cells are visible (arrow in A) as well as scattered cells in the neuropil, which are presumably glial cells (arrowheads in A). In larvae, Drostar1 signals were detected in few cell bodies in the central nervous system (arrowheads). C, section of a larvae. Immunostaining of cells in brain (arrowheads) and corpora allata (ca) using Manduca pGlu-allatostatin C antibodies. lam, laminar; bars, 50 μm.

Fig. 8. Expression analysis of Drostar1 and -2 and localization of their agonist. In situ hybridization was performed with probes specific for Drostar2 (A) or Drostar1 (B) on sections from adult Drosophila heads (A) or larvae (B). Specific hybridization was detected in the optic lobe (ol) and the pars intercerebralis (pi). At higher magnification of optic lobe, numerous labeled laminar monopolar cells are visible (arrow in A) as well as scattered cells in the neuropil, which are presumably glial cells (arrowheads in A). In larvae, Drostar1 signals were detected in few cell bodies in the central nervous system (arrowheads). C, section of a larvae. Immunostaining of cells in brain (arrowheads) and corpora allata (ca) using Manduca pGlu-allatostatin C antibodies. lam, laminar; bars, 50 μm.

TABLE II
Sequence comparison and agonistic activity of insect allatostatin peptides (AI) and mammalian agonists for somatostatin/opioid receptors

| ligand      | sequence                          | signal |
|-------------|-----------------------------------|--------|
| Dm Gln-A1C  | QVRRTAVHFPIKSV-OM                 | yes    |
| Dm pGlu-A1C | <EVRTAVHFPIKSV-OM                | yes    |
| SST-14      | AGKNNFWRKTFTSC-OM                 | no     |
| Met-enkephalin | YGGFM-OM                        | no     |
| Leu-enkephalin | YGGFL-OM                      | no     |
| Dm A1-A     | SRPYSFGGL-NH₂                    | no     |
| Dp A1-A7    | APSCAQLRGLYFGGL-NH₂              | no     |

All ligands were tested at a concentration of 1 μM in Xenopus oocytes injected with Drostar1 or -2 and GIRK1. Dm, D. melanogaster; Dp, Diploptera punctata.

and specificity. As depicted in Table II, the natural agonists of Drostars, pyro-Glu- and Gln-allatostatin C, bear only superficial similarity to somatostatin-14, since they are rather similar in their molecular weights (14 versus 15 amino acid residues) and also in carrying a disulfide loop absent in all other known allatostatin peptides and essential for high affinity to the Drostar receptors. Otherwise, there is no detectable sequence similarity between the allatostatin C peptides and SST-14.

The affinity and ligand specificity of mammalian somatostatin receptors are determined by two sites; one consists of a single aspartate residue in transmembrane region 3 (TM3; Asp124 in SSTR3) (13), and the other consists of a region between TM6 and -7 facing the extracellular side of the plasma membrane (14). The aspartate residue of the SSTR is part of a narrow and selective pocket that forms an ion pair with the positively charged lysine residue at position 4 or 9 of somatostatin (15). In the somatostatin-like receptors of Drosophila, Drostar1 and 2, this aspartate is replaced by threonine (Thr140). When converting this residue into an aspartate by site-directed mutagenesis, the recombinant Drostar1 receptor showed a 3-fold reduced affinity for pyro-Glu-allatostatin C; no activity was observed with somatostatin 14. A Drostar1 chimeric mutant carrying the second binding pocket of SSTR2 from the middle of TM6 to the middle of TM7 was activated by neither pyro-Glu-allatostatin C nor somatostatin 14 at all concentrations tested. Accordingly, there is no cross-reactivity between the Drostar and the SSTR ligand/receptor systems even in the mutant constructs. This suggests that during evolution of this group of receptors, a common scaffold of a receptor protein coupled to inhibitory G-proteins appears to be maintained, whereas the extracellular parts facing the ligand are rather variable in order to accommodate different peptide ligands. Both SSTRs and Drostars presumably use part of TM3 and the extracellular portions of TM6 and TM7 to accommodate their agonists, as is underlined by our mutagenesis study.

Since the Drostars are the closest relatives of mammalian SST/opioid receptors but are not activated by these peptides (Table II), it may be speculated that neither opioid nor SST-like peptides exist in Drosophila. This view is supported by our inability to identify SST or opioid precursor-like sequences in the Drosophila genome database (data not shown), although exon-intron boundaries within a predicted peptide may complicate such a search. As a conclusion, whereas many receptors from vertebrates have a structural analog in invertebrates (6), the latter may use an entirely different set of peptides to activate these receptors. For a functional annotation of the many receptor sequences generated in genome projects such as

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2 M. Heckmann, personal communication.
those for *D. melanogaster* or *Caenorhabditis elegans*, it may therefore in many cases become necessary to identify the physiological ligands of novel receptors by the reverse pharmacology approach that has been used here.

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