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## 2 **The TRH-ortholog EFLamide in the migratory locust**

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16

## Abstract

18 Arthropod EFLamide genes in chelicerates, myriapods, decapods and non pterygote hexapods  
encode various EFLamide paracopies on a single precursor. However, in more advanced insect  
20 species such multiple EFLamide paracopies encoding genes are absent. In some Hemiptera  
putative exons of an EFLamide gene coding for a single EFLamide have been identified, while in  
22 the migratory locust a similar exon could potentially code for two EFLamide peptides. The  
recent identification of an EFLGamide from *Platynereis dumerilii* as the ligand for an ortholog  
24 of the TRH GPCR, suggested that the arthropod EFLamides might similarly activate TRH GPCR  
orthologs. We here identify the TRH GPCR ortholog from *Locusta migratoria* and show that it is  
26 activated in nanomolar concentrations by the two EFLamides previously predicted from this  
species. We also show that in the central nervous system there seems to be only a single bilateral  
28 neuron in the protocerebrum expressing this peptide. Given this very limited expression of  
EFLamide in locusts, it is perhaps not surprising that this gene and its receptor have been lost in  
30 many other insect species. This shows again that although neuropeptides and their receptors may  
persist in different evolutionary lineages, their functions can change dramatically.

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Key words: *Locusta migratoria* – TRH – EFLamide – GPCR – evolution

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## 1. Introduction

40 Many neuropeptides and their receptors originated very early during evolution and as a  
consequence their descendant vertebrate neuropeptides have orthologs not only in invertebrate  
42 deuterostomes, but also in protostomes (Mirabeau and Joly, 2013). In some cases both the  
structures of the ligand-receptor combinations and their functions show some similarity between  
44 the two major bilaterian lineages, but often the functions of what appear to be orthologs are  
distinctly different. For example, there is little evidence that the insect homologs of  
46 gonadotropin-releasing hormone are involved in the regulation of reproduction.

One such well known vertebrate neuropeptide is TRH (thyroid stimulating hormone  
48 (TSH)-releasing hormone), the peptide that forms part of the TRH-TSH-thyroid hormone  
feedback loop of the hypothalamus-pituitary-thyroid axis. The first indication for the existence of  
50 an insect TRH related peptide came from the identification of an ortholog of the TRH GPCR in  
the transcriptome from the brown planthopper *Nilaparvata lugens*, but there was no obvious  
52 ligand (Tanaka et al., 2014). Deorphanization of a TRH receptor ortholog from the annelid  
*Platynereis dumerilii* revealed its ligand to be FSEFLGamide (Bauknecht & Jékely, 2015). This  
54 peptide seems to be orthologous to the arthropod EFLamides, which were first described from  
chelicerates, where the genes have two different transcripts producing either EFLamides or  
56 EFLGGPamides (Veenstra, 2012), while some spiders have a second gene that produces  
exclusively a single EFLGGPamide (Veenstra, 2016a). The owl limpet *Lottia gigantea* and the  
58 annelids *Helobdella robusta*, *Capitella teleta* and *Platynereis dumerilii* have orthologous genes  
that similarly show alternative splicing (Veenstra 2010, 2011; Conzelmann et al., 2013).  
60 Although the alternative splicing of this gene thus likely predates the separation of the arthropods

and thelophotrochozoans, in pancrustaceans and the centipede *Strigamia maritima* EFLamide  
62 genes appear to have only a single transcript (Veenstra, 2016a,b).

EFLamide genes coding multiple EFLamide paracopies are present in apterygote  
64 hexapods (Derst et al., 2016) and in the genome of the mayfly *Danica ephemera*, but in more  
advanced insect species only partial putative cDNA or genomic sequences have been found.  
66 These include various hemiptera, including the bed bug *Cimex lectularius* (Predel et al., 2018),  
but also *Nilaparvata lugens*, the species with a GPCR similar to the TRH receptor, but also a  
68 dragonfly, a damselfly and the migratory locust (Veenstra, 2019). Some have questioned whether  
a small DNA sequence containing a sequence that could code for a single EFLamide is sufficient  
70 evidence for the existence of such a peptide. Indeed, some DNA sequences suggesting the  
existence of a neuropeptide gene can be misleading (Veenstra, 2017). So we felt it would be  
72 interesting to see whether we could get more convincing evidence for an EFLamide signaling  
system in insects. We choose to do this in the migratory locust *Locusta migratoria*, a species  
74 with a well studied neuropeptidome. Furthermore, in the locust the putative EFLamide precursor  
still has two EFLamide copies, which suggests that EFLamide may be physiologically more  
76 important in this species than in those in which the gene codes for only a single paracopy.

We prepared antisera to both EFLamide and its putative precursor and used these to  
78 provide evidence that in the locust this neuropeptide gene is only expressed in a single bilateral  
brain neuron. In order to show that EFLamide is a functional neuropeptide in this species we  
80 then identified the locust homolog of the TRH GPCR and showed that it is activated at  
nanomolar concentrations of the previously predicted *Locusta* EFLamides.

82

## 2. Materials & Methods

### 84 2.1. Insects

86 *Locusta migratoria* and the two-spotted cricket *Gryllus bimaculatus* were obtained from  
a local pet shop, the American cockroach *Periplaneta americana* was from a small laboratory  
culture started from animals gifted by Peter Kloppenburg (Köln, Germany). The common stick  
88 insect *Carausius morosus* was raised on blackberry leaves from eggs purchased on e-bay from  
Spain.

90

### 2.2. Peptides

92 The following peptides were custom synthesized by PhtdPeptides Co, Ltd (Zhengzhou  
City, China) : *Procambarus* AMGSEFLamide (95% purity), *Locusta* EFLamide-1 (NLGSEFL-  
94 amide, 95% purity), *Locusta* EFLamide-2 (MENLGSEFL-amide) 95% and *Locusta* EFLamide  
precursor peptide (CMQQSTTYNDLVQFRVV, 85% purity). The first of these corresponds to  
96 an EFLamide peptide predicted from the red swamp crayfish *Procambarus clarkii* (Veenstra,  
2016) and the others are the predicted mature *Locusta* EFLamides as well as part of the predicted  
98 *Locusta* EFLamide precursor (Fig. 1). TRH (TSH releasing hormone) was from Genscript  
(Leiden, Netherlands).

100

### 2.3. EFLamide antisera

102 Three different polyclonal antisera were produced, a rabbit and a mouse antiserum to  
AMGSEFLamide conjugated to bovine serum albumen (BSA) through its N-terminal using  
104 Difluorodinitrobenzene as described (Tager, 1976) and a rabbit antiserum to part the putative

*Locusta* EFLamide precursor (Veenstra, 2019). For the latter CMQQSTTYNDLVQFRVV was  
106 conjugated through its N-terminal cysteine to BSA using sulfo-SMCC (Sulfosuccinimidyl-4-[N-  
maleimidomethyl]cyclohexane-1-carboxylate). Antisera were produced by Pineda Antikörper-  
108 Service (Berlin, Germany).

#### 110 2.4. Immunohistology

Insects were dissected and fixed in 4% paraformaldehyde in PBS for 1 to 4 hrs at room  
112 temperature. After eight 30 minute washes in PBSAT (PBS containing 0.1% sodium azide and  
1% Triton X-100) tissues were incubated in 10% normal goat serum for one hour, followed by  
114 incubation in primary antiserum for three to six days. Tissues were washed eight times for 30  
minutes in PBSAT and incubated for one hour in 10% normal goat serum. This was followed by  
116 incubation in secondary antiserum for one night to three days. Tissues were again washed eight  
times for 30 minutes and then transferred to 20% glycerol for 15 minutes. Incubation for 15  
118 minutes in 40%, 60% and 80% glycerol followed and tissues were then mounted in 80% glycerol.  
All washes and antibody incubations were done at room temperature under gentle shaking.  
120 Primary antiserum dilutions were 1:2000 for the mouse anti-EFLamide, 1: 8,000 for the rabbit  
EFLamide and 1: 8,000 for the EFLamide precursor antiserum. Other primary antisera used were  
122 anti-corazonin (Veenstra, 1991), anti-leucokinin IV antiserum (Chen et al., 1994) and an anti-  
Crustacean Hyperglycemic Hormone (CHH) from the crayfish *Orconectes limosus* (Keller,  
124 1988); all these antisera were raised in rabbits. DyLight-488-conjugated goat anti-mouse IgG and  
DyLight-549-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Europe, UK) were used  
126 as secondary antibodies at a 1:1,000 dilution. Incubations in primary antisera were done for three

to seven days and secondary antisera for two to three days. Preabsorption experiments were  
128 performed using 10 nmol of peptide per ml of diluted antiserum.

### 130 2.5. Bioinformatics

Transcripts for neuropeptides and GPCRs were produced as described in detail elsewhere  
132 (*e.g.* Veenstra and Khammassi, 2017) essentially using the SRA Toolkit  
(<https://www.ncbi.nlm.nih.gov/sra/docs/toolkitsoft/>) and Trinity (Grabherr et al., 2011). A listing  
134 of the various SRAs used is provided as supplementary data (page 2). A phylogenetic tree was  
made for the EFLamide and TRH GPCRs using only the conserved parts of the receptors, *i.e.*  
136 from the start of transmembrane region (TM) 1 through the end of TM 5 and then from the  
beginning of TM6 through the end of TM7. Sequence alignment was done using Clustal Omega  
138 (Sievers et al., 2011) on the desktop and was manually inspected using Seaview (Gouy et al.,  
2010). Seaview was also used for selecting the conserved protein regions used for making the  
140 trees using PhyML (Guindon and Gascuel, 2003).

### 142 2.6. Putative EFLamide precursors and receptors

Initial attempts to find the *Locusta* ortholog of *Nilaparvata* GPCR A45 (Tanaka et al.,  
144 2014) from the public *Locusta* SRAs were unsuccessful. We therefore tried to find it using the  
public SRAs from three *Gryllus* species – *G. texensis*, *G. bimaculatus* and *G. pennsylvanicus*.  
146 Incomplete and very similar sequences were obtained from all three species (Supp. Data, page  
4). These sequences were then used in an attempt to find the EFLamide receptor (EFLaR) exons  
148 in the genome of *Locusta migratoria*. However, neither the published genome (Wang et al.,





(Cambridge, ON Canada) and after addition of a Kozak site synthesized by the same company  
172 and cloned in pcDNA3.1(+).

Partial putative EFLamide precursors have been described from a number of insect  
174 species, including *L. migratoria* (Veenstra, 2019). Attempts were made to obtain the complete  
coding sequences using the same methodology as that described above for the putative  
176 EFLamide receptors.

## 178 2.7. Receptor assays

Transient coexpression of the *Locusta* EFLamide receptor with the human cytoplasmic  
180 aequorin reporter (Vernon and Printen, 2002) in Chinese hamster ovary (CHO-K1, Sigma) cells  
was used for the monitoring of intracellular calcium mobilization-triggered bioluminescence  
182 upon activation of the receptor (Simo et al 2011, 2013). The assays were performed in opaque  
96-well microplates (Nunc) using the Fluostar Omega microplate reader (BMG Labtech). The  
184 cells were simultaneously co-transfected by the pcDNA3.1(+)/EFLaR, pcDNA3/Zeo(+)/human  
cytoplasmic aequorin and pcDNA3.1(+)/wild type human G protein alpha 15 subunit G $\alpha$ 15(16)  
186 (cDNA Resource Centre, Bloomsburg, University of Pennsylvania) constructs. The use of  
chimeric G $\alpha$ 15(16) subunit is advocated by its high effectivity to link the calcium mobilization  
188 signaling pathway to the transfected Gi/o coupled receptors (Offermans and Simon, 1995; Park  
et al., 2002, 2003). The cells were preequilibrated with coelenterazine-h (Promega) for the 3h in  
190 room temperature. Various doses of agonist ligands in 50  $\mu$ L were plated in each well followed  
by the injection of the 50  $\mu$ L cell suspension (~15,000 cells). Immediately after the injections, the  
192 changes in luminescence were monitored every 0.5 second during 25 seconds interval and their

integrated values over time were normalized to the largest positive control response in each plate  
194 after background subtractions. The data obtained were analyzed with Excel 16.16.13 (Microsoft  
Office) and the dose response curves including the half maximum response values ( $EC_{50}$ ) were  
196 calculated using Prism 5 software package (GraphPad Software, San Diego California USA).

The negative control consisted of cells transfected with the reporter construct aequorin  
198 and  $G\alpha_{15}(16)$  only, which did not show any response to the ligands tested. In addition the  $10\mu\text{M}$   
concentration of *Ixodes scapularis* myoinhibitory peptide 2 and *Drosophila melanogaster*  
200 SIFamide were used as another negative control for transfected cells.

## 202 **3. Results**

### 204 *3.1. Immunohistology*

Antisera against EFLamide recognizes two different cell types in the brain of the  
206 migratory locust (Fig. 2A,B). Most prominently there is a bilateral interneuron located laterally  
below the calyx that innervates a large number of different areas in the brain. Of these the lower  
208 divisions of the anterior optic tubercles and the ventral layer of the anterior lobe of the lobula are  
very prominently innervated. Other easily recognizable areas are the central body and lower units  
210 of the anterior optic tubercles, while a very fine axon is often found innervating the olfactory  
glomeruli in the deutocerebrum (Fig. 2A).

212 At two- to four-fold higher concentrations there are lateral neuroendocrine cells that  
project to the corpora cardiaca. The latter cells are located close to but different from the  
214 corazonin neuroendocrine cells (Fig. 2B) and similarly project through the nervus corporis

cardiaci II to the corpus cardiacum. The EFLamide antisera also recognize neuroendocrine  
216 lateral cells in *Carausius morosus* and in that species those cells are strongly immunoreactive  
(Fig. 2C). In the American cockroach, *Periplaneta americana*, the bilateral pair is missing and  
218 the lateral neuroendocrine cells are also strongly immunoreactive (Fig. 2D). Like the various  
RFamide peptides, EFLamide has both an aromatic and a charged amino acid in its C-terminal.  
220 Antisera to RFamides are known to easily cross-react with other neuropeptides sharing just a C-  
terminal RFamide or even RYamide (*e.g.* Veenstra and Schooneveld, 1984; Veenstra and  
222 Khammassi, 2017). Given the absence of any trace of an EFLamide receptor in the *Periplaneta*  
genome (Li et al., 2018) or the various brain transcriptome SRAs, it seemed likely that the  
224 immunoreactivity in the lateral neuroendocrine cells was due to cross-reactivity with a different  
neuropeptide. Some of these lateral cells were also immunoreactive with a leucokinin antiserum  
226 and all of them were recognized by an antiserum to CHH from *Orconectes limosus*. As this  
antiserum is known to recognize its insect ortholog ion transport peptide (ITP; Dircksen, 2009),  
228 it seemed plausible that both the EFLamide antisera also recognize at least one of the ITP  
isoforms. Analysis of brain transcriptome SRAs from *P. americana* and *Carausius morosus*  
230 revealed the short form of ITP from this species to have a C-terminal EFLamide, while in  
*Locusta* the C-terminal has an EILamide sequence (Table 1). This convincingly shows that the  
232 presence of EFLamide immunoreactivity in neurons does not prove that such neurons express  
EFLamide. We therefore made another antiserum to part of the previously identified putative  
234 *Locusta* EFLamide precursor (Veenstra, 2019). This antiserum only labels the the bilateral  
neuron in the brain (Fig. 2E), thus suggesting that this is the only neuron that expresses  
236 EFLamide in *Locusta*. No other immunoreactive EFLamide or EFLamide precursor neurons

were found in the nervous system. Immunoreactive enteroendocrine cells were neither found and  
238 although we attempted to find EFLamide immunoreactivity at the periphery, such efforts were  
without success.

240 Immunoreactivity was reduced but not completely abolished after preincubation of the  
EFLamide antisera with each of the three EFLamide peptides. The immunoreactivity of the  
242 antiserum to EFLamide precursor was similiary diminished after preincubation with the peptide  
used to make the antiserum.

244

### 3.2. EFLamide precursors

246 Various attempts were made to use the transcriptome SRAs from three *Gryllus* species  
and *L. migratoria* to construct complete cDNAs for the putative EFLamide precursors. The  
248 results of these attempts resemble those obtained after similar efforts for RYamide precursor  
transcript from *Drosophila melanogaster* (Veenstra and Khammassi, 2017). Thus, one can find  
250 the exon coding the EFLamide peptide(s), but exons upstream are not identified. Those RNAseq  
reads that contain sequence 5' to the coding exons either contain in frame stop codons (*Gryllus*)  
252 or coding sequences from a different protein (*Locusta*). In the case of the locust some Trinity  
produced transcripts were obtained that at first sight looked credible as they have a signal  
254 peptide. However, the RNAseq reads that had been added to the putative EFLamide precursor  
reads belong to the *Locusta* ortholog of the protein first identified from honeybees as  
256 IDLSRFYGFHNT-containing or prohormone 4 (Hummon et al., 2006). The latter protein is very  
well conserved (Suppl. Data, page 3), and analysis of RNAseq data show that the very large

258 majority of RNAseq reads support a typical IDLSRFYGHFNT-containing ortholog and hence  
this transcript has to be an artifact.

260

### 3.3. *EFLamide receptor*

262 It should probably not come as a surprise that when it is difficult or impossible to  
construct the cDNA sequence for the neuropeptide that the one for its receptor is also  
264 complicated. In the end a complete transcript could be constructed by combining RNAseq  
derived partial transcripts with genomic sequences and a PCR product. The translated sequence  
266 of the *Locusta* EFLamide receptor cDNA is predicted to have a signal peptide in addition to  
seven transmembrane regions typical of a GPCR.

268 Together with public and ad hoc constructed orthologs and a few vertebrate TRH GPCRs  
(for complete protein sequences and alignment see Suppl. Data, pages 5 and 10 respectively) a  
270 phylogenetic tree was constructed that was rooted using the A isoform of the *Drosophila* ETH  
receptor (the *Drosophila* GPCR that is most closely related to the EFLamide receptor). The  
272 results show the arthropod EFLamide GPCRs as a single cluster in which the branch length is  
considerably smaller in the chelicerates than in insects and crustaceans, suggesting that in the  
274 latter species natural selection to maintain the sequence of this receptor is less intense than in  
chelicerates.

276

### 3.4. *EFLamide receptor activation*

278 The two predicted EFLamide peptides from *L. migratoria* were highly active on the  
EFLamide receptor, with EC<sub>50</sub> values of 130.2 nM and 138.3nM for *Locusta* EFLamide-1 and

280 EFLamide-2 respectively (Fig. 4A). Slightly less robust luminescent responses were also  
generated by *Procambarus* EFLamide with an EC<sub>50</sub> value of 532.3 nM (Fig. 4A). The EFLamide  
282 receptor was much less sensitive to different doses of TRH with EC<sub>50</sub> value of 1.499 μM, when  
compared to those of tested EFLamide peptides. For all of the active doses of the EFLamide, the  
284 increase in luminescence was observed after ~3 second after the ligand was applied, and this  
luminescence peaked within the 5-12s depending of ligand concentration (Fig. 4B). The ligands  
286 tested in this study showed no activity on cells that were not transfected with EFLamide receptor  
indicating that the luminescent responses were specifically mediated by the transfected receptor.  
288 The cells that expressed EFLamide receptor were activated only by EFLamides and TRH, and  
did not respond to the other tested ligands.

290

#### 4. Discussion

292 Antisera raised to EFLamide recognized two different cell types in the migratory locust,  
one of which was only weakly labeled in this species, but much more intensely in a stick insect  
294 and the American cockroach. The latter cell type produces ITP. One of the ITP isoforms has an  
EFLamide C-terminal in *Periplaneta* and *Carausius*, two species in which the ITP  
296 neuroendocrine cells react strongly with the EFLamide antisera. In *Locusta* on the other hand the  
corresponding ITP isoform has a C-terminal EILamide and the ITP cells are only weakly  
298 EFLamide immunoreactive. This suggested that the EFLamide antisera cross-reacts with these  
ITP isoforms. We thus produced another antiserum to a different part of the *Locusta* EFLamide  
300 precursor. This second antiserum only recognizes the bilateral neuron in the brain and we thus  
conclude that these are the only neurons that express the EFLamide gene in *Locusta*.

302 Antisera to RFamide neuropeptides similarly cross-react with different neuropeptides like  
e.g. FMRFamide and myosuppressin. Although both of FMRFamide and myosuppressin have  
304 their own typical receptor, it has been shown that in the prothoracic gland of *Bombyx mori* the  
myosuppressin receptor in this tissue can be activated by both myosuppressin, presumably as a  
306 hormone, and FMRFamide as a neuromodulator directly released in the tissue by efferent  
neurons (Yamanaka et al., 2005, 2006). This raises the question whether there might be similar  
308 interactions between EFLamide and the short isoforms of ITP. Although this may seem  
speculative, it has already been demonstrated that in *Bombyx* the tachykinin receptor can be  
310 activated by both the long form of ITP and tachykinins (Nagai et al., 2014; Nagai-Okatani et al.,  
2016).

312 To support the argument that despite its very limited expression the EFLamide  
neuropeptide is functional in the locust we identified its putative receptor based on previous  
314 work in the annelid *Platynereis dumerilii* that showed that in this species a TRH GPCR ortholog  
is activated by EFLGamide (Bauknecht and Jékely, 2015). The scarcity of RNAseq reads in the  
316 various *Locusta* transcriptome SRAs and the difficulty of amplifying this GPCR by RT-PCR  
suggest that the expression of the receptor like that of the EFLamide precursor is very low.  
318 Nevertheless we were able to show that it is activated at nanomolar concentrations of the  
previously predicted EFLamides from this species.

320 Neuropeptides that are encoded by genes coding multiple structurally similar paracopies  
are typically released into the hemolymph. The potent physiological effects of EFLamide on the  
322 motor patterns produced by the cardiac and stomatogastric ganglia in the lobster *Homarus  
americanus* (Dickinson et al., 2019) suggests that in Decapods EFLamide is likely released into



324 the hemolymph as a hormone. Such multiple paracopies coding EFLamide genes are also present  
in non-apterygote hexapods and mayflies, but in those advanced insects that still have this gene  
326 the number of paracopies is reduced to only one or two (Derst et al., 2016; Veenstra, 2019). This  
in combination with a relatively high  $EC_{50}$  value for the interaction of the peptides with their  
328 receptor in *Locusta* suggests that in the latter species, the peptide may have lost its hormonal  
function. The presence of only a single bilateral interneuron in the central nervous system that  
330 expresses the EFLamide gene reinforces this hypothesis.

The bilateral pair of ELFamide-cells in the locust brain have unusually wide  
332 ramifications in superior protocerebral brain areas. Interestingly, the areas innervated most  
densely, the lower layer of the anterior lobe of the lobula, the lower units of the anterior optic  
334 tubercles, and the central body and lateral complex are all involved in the processing of skylight  
polarization signals used for spatial orientation of the locust (Homberg et al., 2003, 2011). It is  
336 therefore, possible that the ELFamide neurons exert a modulatory input onto the navigation  
system of the locust by simultaneous targeting several stages of the sky compass system in the  
338 locust brain.

Concentrations of neuropeptides are much lower in the hemolymph than locally inside  
340 the nervous system. It is therefore not surprising that the  $EC_{50}$  values of around 130 nM for the  
two *Locusta* EFLamides are higher than those typically found for neuropeptides released into the  
342 hemolymph and one would expect these values to be significantly lower in species where the  
peptides still function as hormones. This suggests that the evolutionary constraints on  
344 maintaining peptide and receptor structures will be relaxed once a neuropeptide is released  
exclusively within the central nervous system and no longer used as a neurohormone. The long

346 branch length of the *Locusta* EFLamide GPCR in the phylogenetic tree (Fig. 3) indeed suggests  
that this might be the case here. It is interesting to note that the branch length of the *Nilaparvata*  
348 GPCR on this tree is even longer and that its apparent place on the phylogenetic tree does not  
correspond to its true phylogenetic position, thus suggesting that here the evolutionary constraint  
350 on maintaining the receptor is still much weaker. Indirectly, it suggests that EFLamide in  
*Nilaparvata* is not released in large quantities.

352 Our results convincingly show that the EFLamide neuropeptide is expressed in a very  
small number of neurons. This no doubt explains that it has never been physically identified as  
354 such in insects. Nevertheless, the identification of a *Locusta* EFLamide receptor that is activated  
by nanomolar concentrations of two predicted EFLamides must lead to the conclusion that this  
356 neuropeptide signaling system is still functional in this species. However, this very limited  
expression of this neuropeptide also suggests that whatever its original function as an arthropod  
358 neurohormone may have been, that function must have become superfluous as insects evolved.

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**Table 1**

516 Peptide sequences. Sequences of the two predicted EFLamides from *Locusta migratoria*  
(Veenstra, 2019). For comparison the *Procambarus* EFLamide used as antigen for the EFLamide  
518 antiserum as well as the C-termini of the short isoforms of ion transport peptide from *L.*  
*migratoria*, *P. americana* and *C. morosus* are also provided. Note that these peptides show  
520 sufficient structural similarity to EFLamide to explain the cross-reactivity of the EFLamide  
antiserum with these ion transport peptides. The peptide used to make a *Locusta* EFLamide  
522 precursor antiserum is also shown to demonstrate that it has no sequence similarity to EFLamide.

524	<i>Locusta</i> EFLamide-1	NLGSEFLamide
	<i>Locusta</i> EFLamide-2	MENLGSEFLamide
526	<i>Procambarus</i> EFLamide	AMGSEFLamide
	C-terminal of <i>Locusta</i> ITP	---KFNQMVEILamide
528	C-terminal of <i>Periplaneta</i> ITP	---KFNQMAEFLamide
	C-terminal of <i>Carausius</i>	---NFSQMVEFLamide
530	<i>Locusta</i> EFLamide precursor peptide	CQQSTTYNDLVQFRVV

532 **Legends to the figures.**

534 **Fig. 1.** Partial genomic sequence of *Locusta migratoria* and its plausible translation into the last  
coding exon of the putative EFLamide gene of this species. Small lettering corresponds to the  
536 DNA sequence, with the untranslated intron and 3'-sequence in italic lower case and the  
translated in bold capitals while the large lettering corresponds to the predicted protein sequence.  
538 The two predicted *Locusta* EFLamides are indicated in light blue, with the Gly residues  
predicted to be transformed in C-terminal amides in dark blue. Predicted convertase cleavage  
540 sites are highlighted in red [processing at the single Arg residue is supported by the presence of  
an Arg residue in the P4 pocket (Veenstra, 2000)]. Yellow highlighting indicates the peptide  
542 used for making *Locusta* EFLamide precursor specific antiserum.

544 **Fig. 2.** Immunohistological localization of EFLamide immunoreactivity. **A.** Adult female  
*Locusta migratoria* brain showing the EFLamide immunoreactive bilateral neuron (1). Note the  
546 strong immunoreactivity in the lower units of the anterior optic tubercles (2) and the ventral layer  
of the anterior lobe of the lobula (3); immunostaining is also prominent in the central body (4)  
548 and lateral complex (5). **B.** EFLamide- (Bi and Bii) and corazonin-immunoreactive cells (Bii and  
Biii) in green and magenta respectively in the pars lateralis of *Locusta*. Note that the  
550 immunoreactivity in the bilateral neuron (in the lower part of the pictures) is much stronger than  
that in the lateral neuroendocrine cells. **C.** EFLamide- (Ci and Cii) and corazonin-  
552 immunoreactive cells (Cii and Ciii) in green and magenta respectively in the pars lateralis of  
*Carausisus morosus*. Note that the immunoreactivity in the EFLamide immunoreactive

554 neuroendocrine cells in this species is much stronger than in *Locusta* (Bi and Bii). **D.** EFLamide-  
(Di and Dii) and CHH-immunoreactive cells (Dii and Diii) in green and magenta respectively in  
556 the pars lateralis of *Periplaneta americana*. Note that the immunoreactivity in the EFLamide  
immunoreactive neuroendocrine cells in this species is much stronger than in *Locusta* and also  
558 note the two antisera recognize exactly the same cells. **E.** EFLamide precursor (Ei and Eii) and  
EFLamide immunoreactivity (Eii and Eiii) in green and magenta respectively in a last instar  
560 *Locusta* larva. Note that the two antisera label exactly the same neurons and axons.

562 **Fig. 3.** Phylogenetic analysis of EFLamide and TRH GPCRs. The Arthropod EFLamide receptor  
clade is highlighted in blue, the vertebrate TRH GPCR clade in yellow. Note that the *Platynereis*  
564 EFLamide receptor seems somewhat more similar to the vertebrate TRH than to the arthropod  
EFLamide receptors. Whereas the branch lengths of the chelicerate EFLamide receptors are  
566 relatively short, those of the other arthropod GPCRs are longer and that the branch lengths for  
the *Locusta* and *Nilaparvata* receptors are the longest. Also note that the location of the  
568 *Nilaparvata* GPCR on the tree does not correspond to its true phylogenetic position, suggesting  
significant relaxation of natural selection to maintain its primary sequence. For individual  
570 sequences and other details see Supplementary Data. PhyML ln(L)=-7073.0 281 sites LG 100  
replic. 4 rate classes.

572

**Fig. 4.** Cellular responses observed after ligand-mediated calcium mobilization in aequorin  
574 reporter assays in CHO-K1 cells that were transfected with EFLamide receptor. (A) The dose  
response curves for *Locusta* EFLamide-1, EFLamide-2, *Procambarus* EFLamide and TRH. (B)

576 Typical representative responses of the cells when treated with different doses of EFLamides  
(*Locusta* EFLamide-2 in this case). Inset in (B) shows the integrated relative luminescent values  
578 (RLU) calculated from 50 intervals within the 25 second responses. The bars in A indicate the  
standard error for a minimum of three replicated plates.

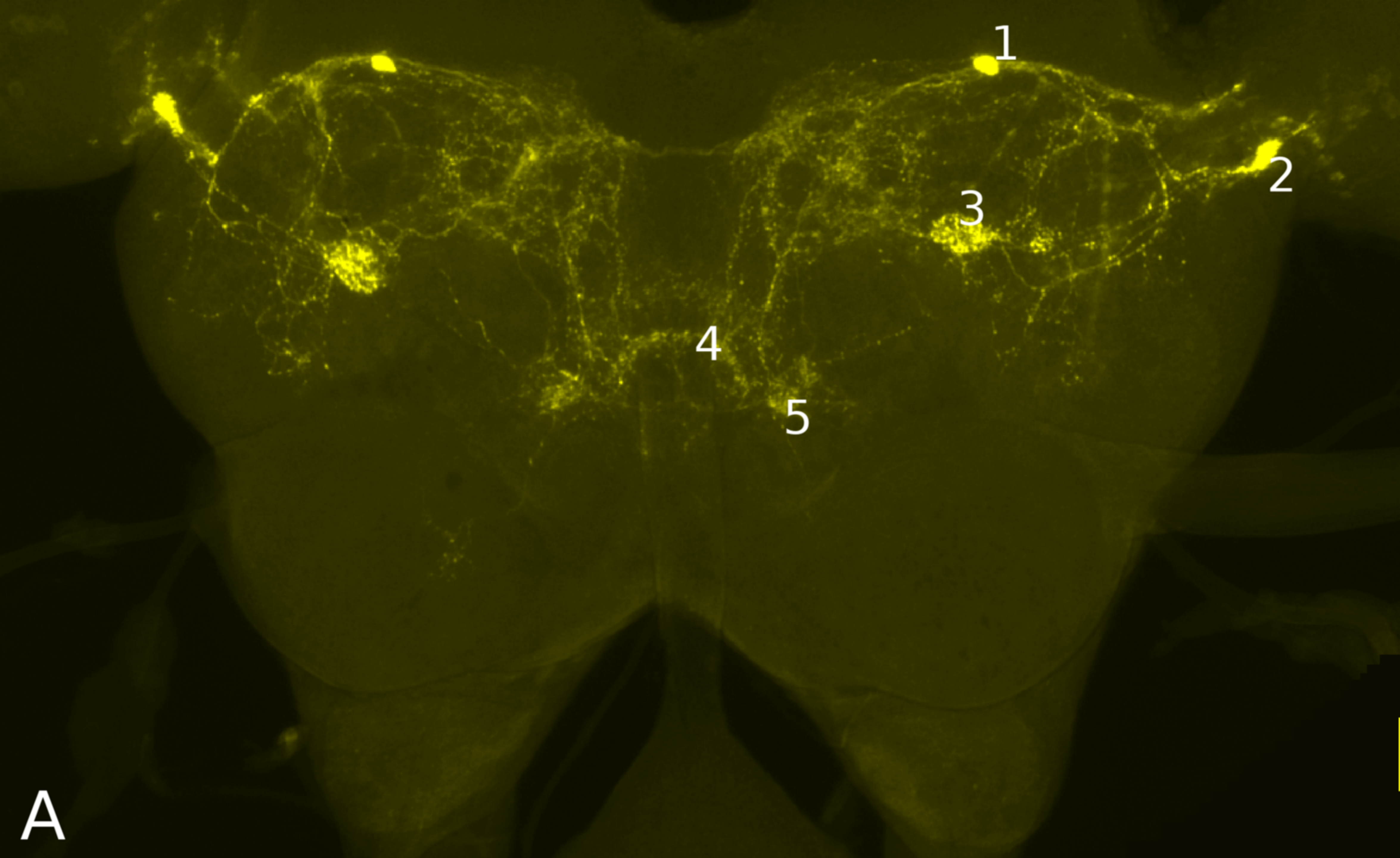
580

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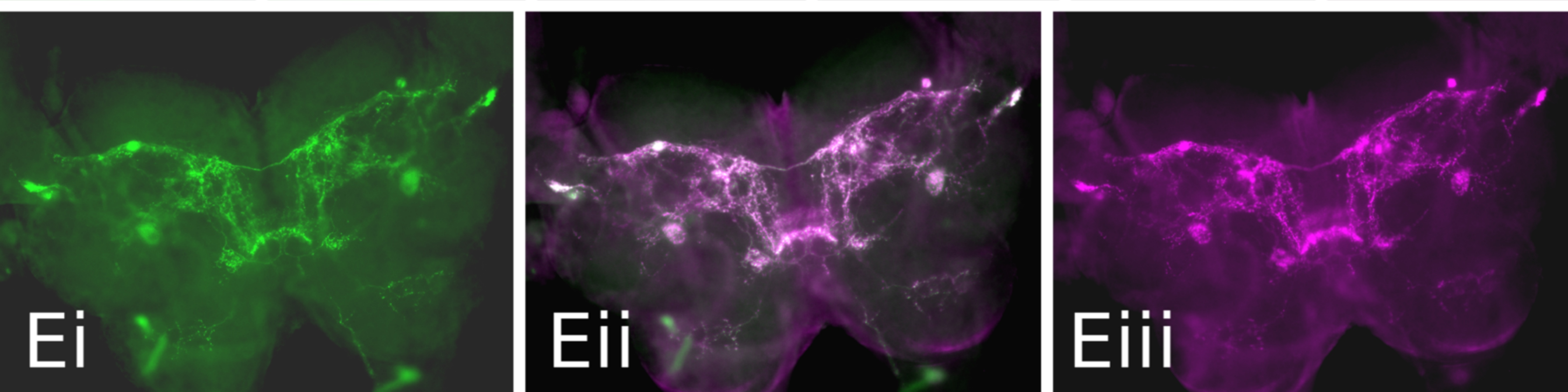
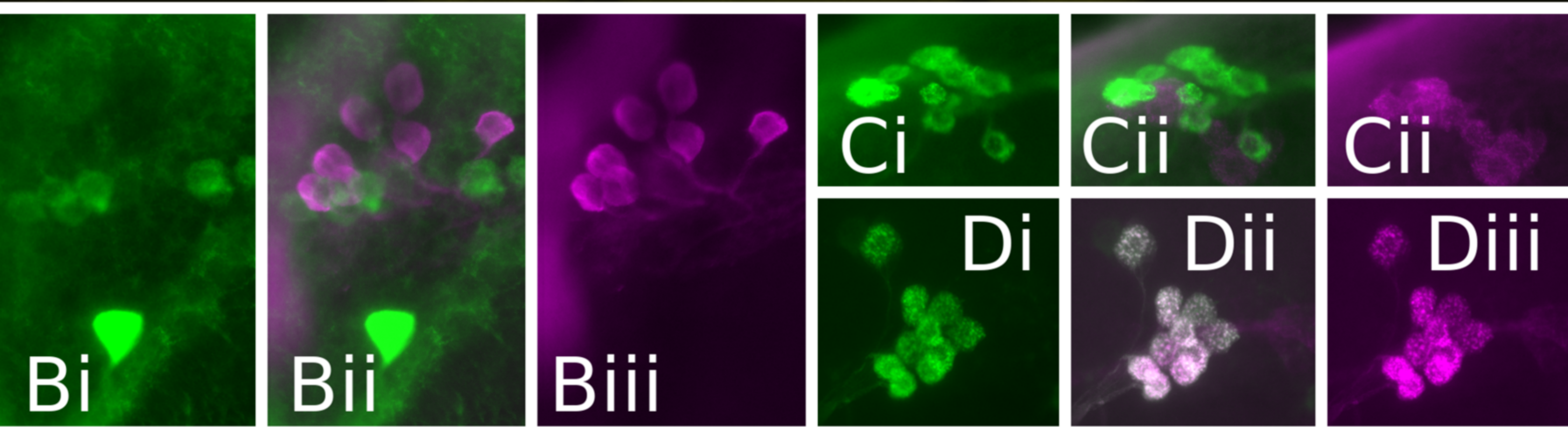
XFLKTLSSLNERLSEM**QQSTTYNDLVQFRVVR**RNL

GGATCCGAATTCCTAGGAAAACGAATGGAAAACCTTGGGATCAGAATTTCTAGGAAAACGAATGCAAAAACCTTGAAAAATATACTAGGAGTCAAA*taatgtaatatgtctcacctgctaca*

GSEFL**GKR**MENLGSEFL**GKR**MQNLKNIILGVK\*



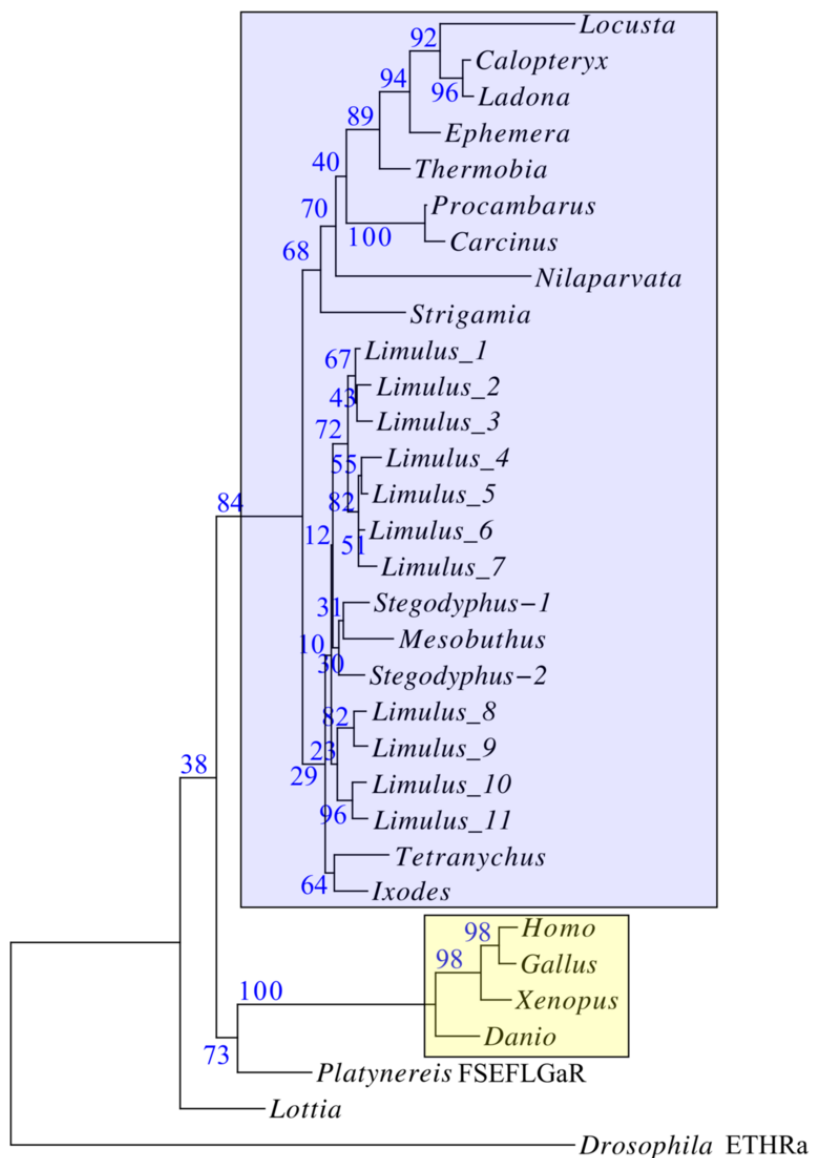
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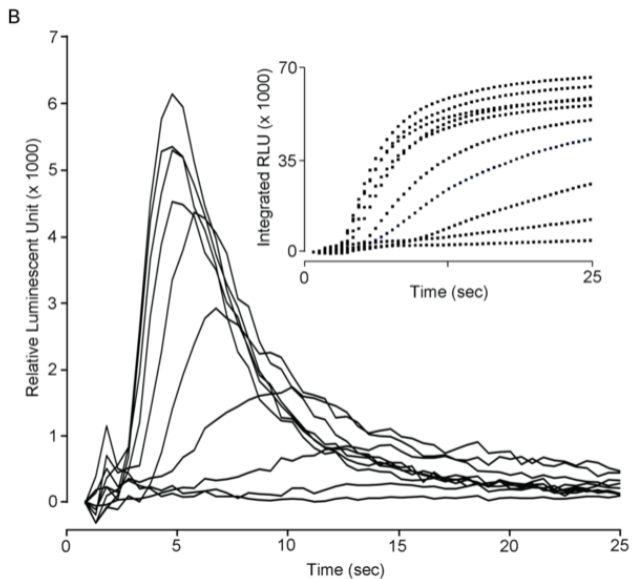
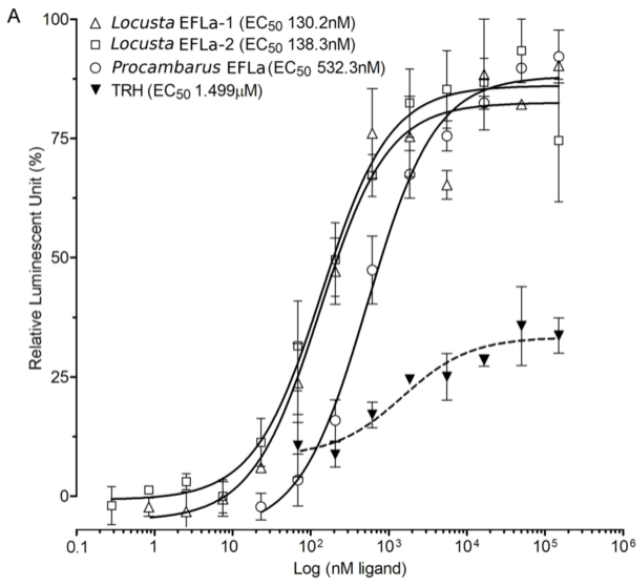
Ei

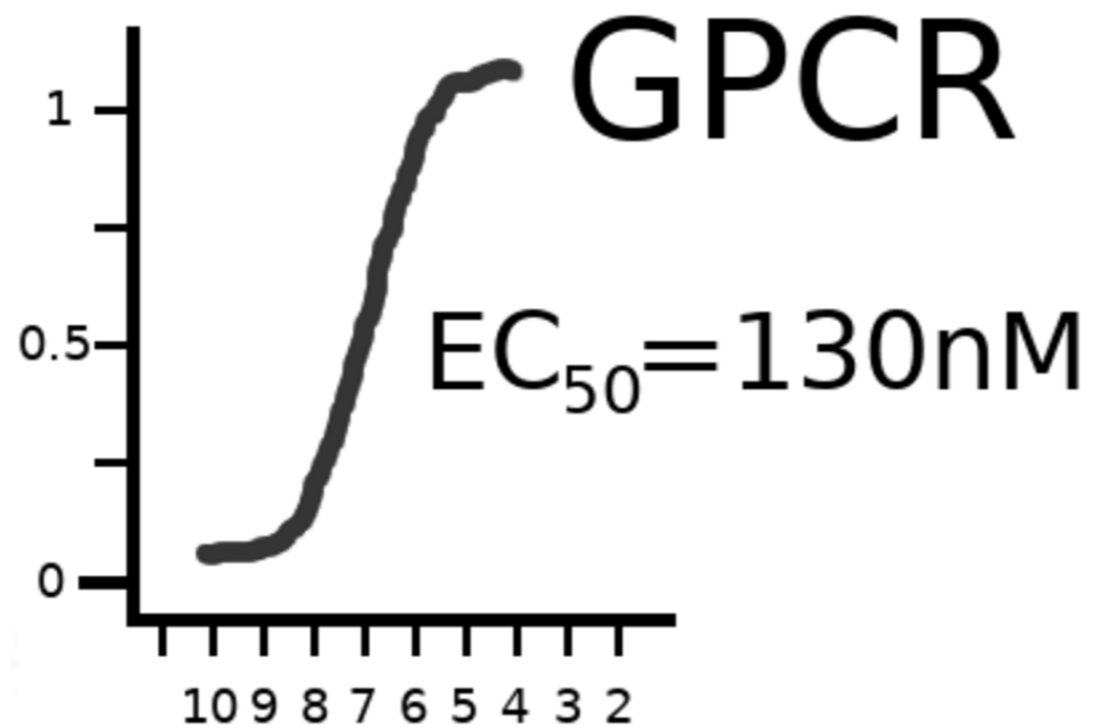
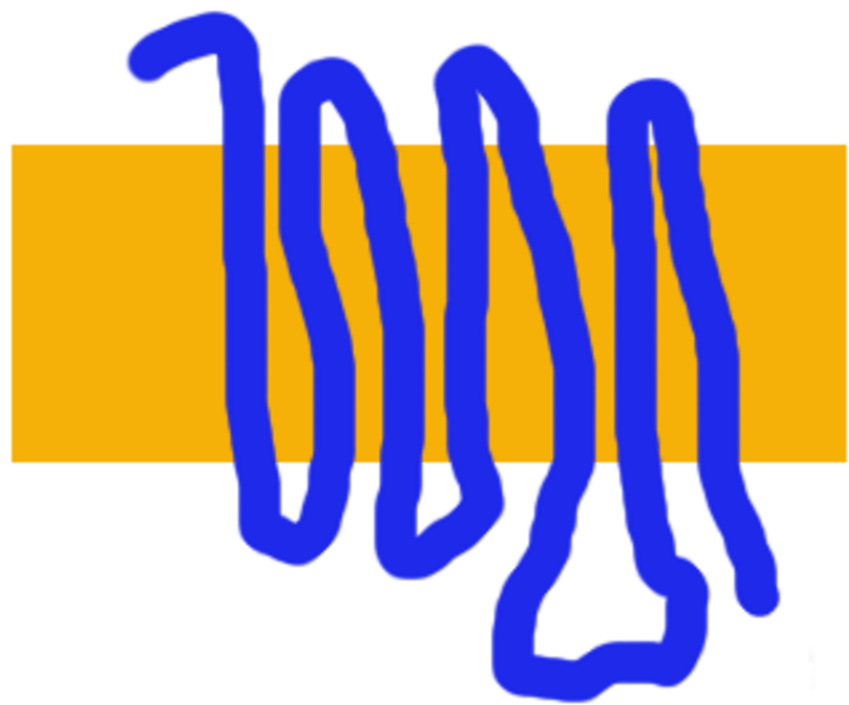
Eii

Eiii









# EFLamide

