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The salivary gland salivation stimulating peptide from *Locusta migratoria* (Lom-SG-SASP) is not a neuropeptide

A salivary gland salivation stimulating peptide was identified from the salivary glands of the migratory locust by its ability to stimulate cAMP production in the same tissue. The gene coding for this peptide has recently been shown to code for a precursor consisting of a signal peptide, several copies of the peptide separated by Lys-Arg doublets and a few other peptides. These data are consistent with it being a neuropeptide. However, antiserum raised to this peptide labels the acini of the salivary glands while RT-PCR only gives positive results in the salivary gland, but not in any ganglion of the central nervous system. Thus, this peptide is not a neuropeptide as previously assumed.

The salivary gland salivation stimulating peptide from *Locusta migratoria* (Lom-SG-SASP) is not a neuropeptide

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20 INTRODUCTION

21

22 Insect neuropeptides are interesting for two different reasons. On the one hand, insects
23 are protostomes, while vertebrates are deuterostomes. Thus, comparing insect with vertebrate
24 neuropeptide regulatory systems provides insight as to how structures and functions may have
25 changed during evolution. On the other hand, many insect species are pests and vectors of
26 disease and thus responsible for serious agricultural damage as well as the transmission of human
27 disease. As insects are becoming increasingly resistant to classical pesticides novel insecticides
28 are constantly needed and it has repeatedly been suggested that agonists or antagonists of
29 insect neuropeptide receptors might offer a solution (*e.g.* Audsley et al., 2015 ; van Hiel et al.,
30 2010). Inhibition or at least disruption of feeding by insects would be very attractive as it would
31 presumably avoid or diminish economic damage, or in the case of disease vectors, might reduce
32 transmission of infectious agents. As production of saliva is usually a first and necessary step in
33 feeding the regulation of salivation by neuropeptides is particularly interesting.

34 A pentadecapeptide was isolated from the salivary glands of the migratory locust by its
35 ability to stimulate the production of cAMP in the same glands at concentration of 10^{-6} M
36 (Veelaert et al., 1995). As it also stimulates salivation (Veelaert et al., 1995), its characteristics
37 suggests it to be neuropeptide that likely acts as a neurotransmitter rather than a hormone. If it
38 were a hormone, one would expect it to stimulate the production of cAMP and salivation in the
39 nanomolar , rather than in the micromolar range and it would be expected in a neurhemal organ,
40 rather than in the salivary gland itself (Veelaert et al., 1995). Unlike most insect neuropeptides
41 orthologs of this peptide have not been identified from any other arthropod, suggesting that it
42 may not be universally present in insects. This might be advantageous as any pesticide based on
43 it could be relatively selective. The genome sequence of the migratory locust (Wang et al., 2014)
44 showed that the predicted precursor encoding this peptide has all the hallmarks of a classical
45 neuropeptide precursor: a signal peptide and a propeptide encoding multiple copies of the
46 peptide separated by Lys-Arg convertase cleavage sites (Veenstra, 2014). It thus appeared of
47 interest to study this putative neuropeptide in more detail, but as reported here the peptide turned
48 out not to be a neuropeptide after all, in spite of the data suggesting otherwise.

49

50 MATERIALS & METHODS

51

52 2.1 Locusts.

53 Adult *Locusta migratoria* were purchased at a local pet store. They were kept for five to
54 seven days at 25 °C and fed fresh grass once a day before being used. Tissues were dissected
55 under saline and either frozen immediately at -80 °C for subsequent RNA extraction, or used for
56 immunohistology.

57

58 2.2 Immunohistology

59 Two mg of SVTVREVGDLFQEWLQQNVN (purity 84 %, Proteogenix, Schiltigheim,
60 France) were conjugated to 5 mg of bovine serum albumin using difluorodinitrobenzene as the
61 conjugation reagent as documented by Tager (Tager, 1976). Polyclonal mouse antisera were
62 raised in three six week-old NMRI female mice as described previously (Veenstra and Ida,
63 2014). Tissues were fixed for 1 to 2 hrs at room temperature. All other immunohistological
64 procedures are the same as described (Veenstra and Ida, 2014). Primary antiserum was diluted 1:
65 500, the secondary antiserum, DyLight-488-conjugated goat anti-mouse IgG that was from
66 Jackson ImmunoResearch Europe (Newmarket, Suffolk, UK), 1:1,000.

67

68 2.3 Bioinformatics.

69 cDNA sequences coding for the *L. migratoria* orthologs of vertebrate PC1 and PC2 are
70 not present in the databases and were therefore obtained by using a combination of the published
71 genome sequence and Trinity on sequences extracted from the various short read archives
72 (SRAs) for this species available at NCBI (SRR014351, SRR014352, SRR058432, SRR058446,
73 SRR058447, SRR058448, SRR058449, SRR058450, SRR058451, SRR058452, SRR058453,
74 SRR058454, SRR058455, SRR058456, SRR058457, SRR058458, SRR058488, SRR058489,
75 SRR058490, SRR058491, SRR058492, SRR058493, SRR058494, SRR058495, SRR058496,
76 SRR058497, SRR058498, SRR058499, SRR058500, SRR058501, SRR058502, SRR058503,
77 SRR1032161, SRR1032192, SRR167712, SRR513208, SRR513209, SRR513210 and
78 SRR513211) using methodology described in detail elsewhere (Veenstra, 2016). Protein and
79 cDNA sequences for *L. migratoria* PC1 and PC2 are provided in the supplementary data.

80

81 2.4 RT-PCR

82 The following tissues were dissected : brain, suboesophageal ganglion, pro- and meso-
83 thoracic ganglia combined, the meta-thoracic ganglia combined with all abdominal ganglia,
84 salivary gland, fat body and midgut. For the analysis of the expression of PC1 and PC2
85 Malpighian tubules and flight muscle were also analyzed. At least two samples were processed
86 completely independently for each of these tissues. Each sample containing tissues from at least
87 four different animals. From salivary glands, fatbody, Malpighian tubules and flight muscle,
88 small parts were taken from four different animals. For the midgut four longitudinal half midguts
89 were processed individually. RNA extraction was performed using mini spin columns from
90 Macherey-Nagel. Next RNA (800-1000 ng) was reverse transcribed in a 20 µl reaction using
91 Moloney Murine Leukemia Virus Reverse Transcriptase (New England Biolabs, Evry, France)
92 and random primers. One µl of the resulting cDNA was next amplified by PCR using OneTaq
93 Quick-Load DNA Polymerase (New England Biolabs) with specific primers for each mRNA.
94 Primers used are : for the salivary gland salivation stimulating peptide: 5'-
95 GCCTTCCTGCTAGTCGTCTG-3 and 5'-TACCTTTTGCCCACTCTTGG-3'; for actin: 5'-
96 AGGGCTGTTTTCCCTCAAT-3' and 5'-GAAGGTGTGGTGCCAGATTT -3'; for PC1: 5'-
97 ACAACCACGTGCACAAGAAG-3' and 5'-TGAATGCGACTAAGCCACAG -3' ; and for PC2:
98 5'-GGTGGACTACCTGGAACACG-3' and 5'-TGTGGATATTCTCCCCAGGT -3' . PCR
99 profiles consisted of 90 second denaturing at 94°C followed by 32 cycles of 30 seconds at 94°C,
100 15 seconds at the annealing temperature and 15 seconds at 68°C the amplification was followed
101 by 5 min extension at 68°C. Annealing temperatures were 55°C, 64°C, 64°C and 60°C for the
102 salivary gland salivation stimulating peptide, actin, PC1 and PC2 respectively. Controls in which
103 water replaced the cDNA showed no PCR amplification.

104

105 **RESULTS & DISCUSSION**

106

107 I anticipated that an antiserum would allow the identification of neurons expressing this
108 peptide. However all three mice produced antisera that labeled the acinar cells of the salivary
109 glands (Fig. 1), while immunoreactive material was completely absent from the central nervous
110 system. This suggested that the peptide was made by the salivary gland itself, rather than by the
111 nervous system. Intron spanning primers were designed to look for expression of the gene coding
112 the peptide and results revealed amplification only in the salivary glands (Fig. 2). The PCR-

113 amplified band was sequenced using the primers for amplification and the sequencing results
114 confirmed the expected sequence (Suppl. Data). Thus there is independent confirmation that this
115 gene is expressed in the salivary gland, but neither in the central nervous system, the fatbody nor
116 the midgut. RT-PCR confirmed strong expression of this gene as (1) amplicons become visible
117 after 20 cycles (Fig. 2) and (2) the slightest contamination of throcacic ganglia with a small piece
118 of salivarygland (these tissues are closely associated with one another) leads to false positives.
119 The integrity of the cDNA samples were checked by looking for expression of actin as a control.

120 The Lys-Arg convertase cleavage sites in the precursor are identical to those commonly
121 found in neuropeptide precursors and thus suggested the presence of a neuropeptide specific
122 convertase in the salivary gland. The cDNA sequences of the two locust orthologs of vertebrate
123 PC1 and PC2 were obtained using Trinity on the various short read archives (SRAs) for this
124 species available at NCBI (Suppl. Data) and intron spanning primers were designed to look for
125 their expression in the salivary gland. PC1 was found not only in the salivary gland, but also in
126 other peripheral tissues (Fig. 3), but PC2 was absent from the salivary glands. It thus appears that
127 the tissue distribution of PC1 in migratory locusts and perhaps other arthropods is much broader
128 than in vertebrates (Seidah et al, 2013).

129 In the last decade a large number of putative neuropeptide genes have been identified in
130 genome sequences of a wide variety of invertebrate species, sometimes based exclusively on the
131 presence of signal peptide and Lys-Arg cleavage sites that separate the presumptive
132 neuropeptides in the precursor. In many cases the predicted peptides show clear homology to
133 known neuropeptides, while in other cases they have subsequently been shown to activate G-
134 protein coupled receptors (*e.g.* Bauknecht et al., 2015). However, as illustrated here the presence
135 of a signal peptide, reputable convertase cleavages sites and multiple copies of the same or a
136 very similar peptide do not make a neuropeptide precursor.

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Figure 1

Immunohistological localization of SG-SASP.

Immunoreactive salivary gland peptide in the adult salivary gland of *L. migratoria*. Note the strong labeling in the acinar cells. Scale bar 250 μm .

*Note: Auto Gamma Correction was used for the image. This only affects the reviewing manuscript. See original source image if needed for review.

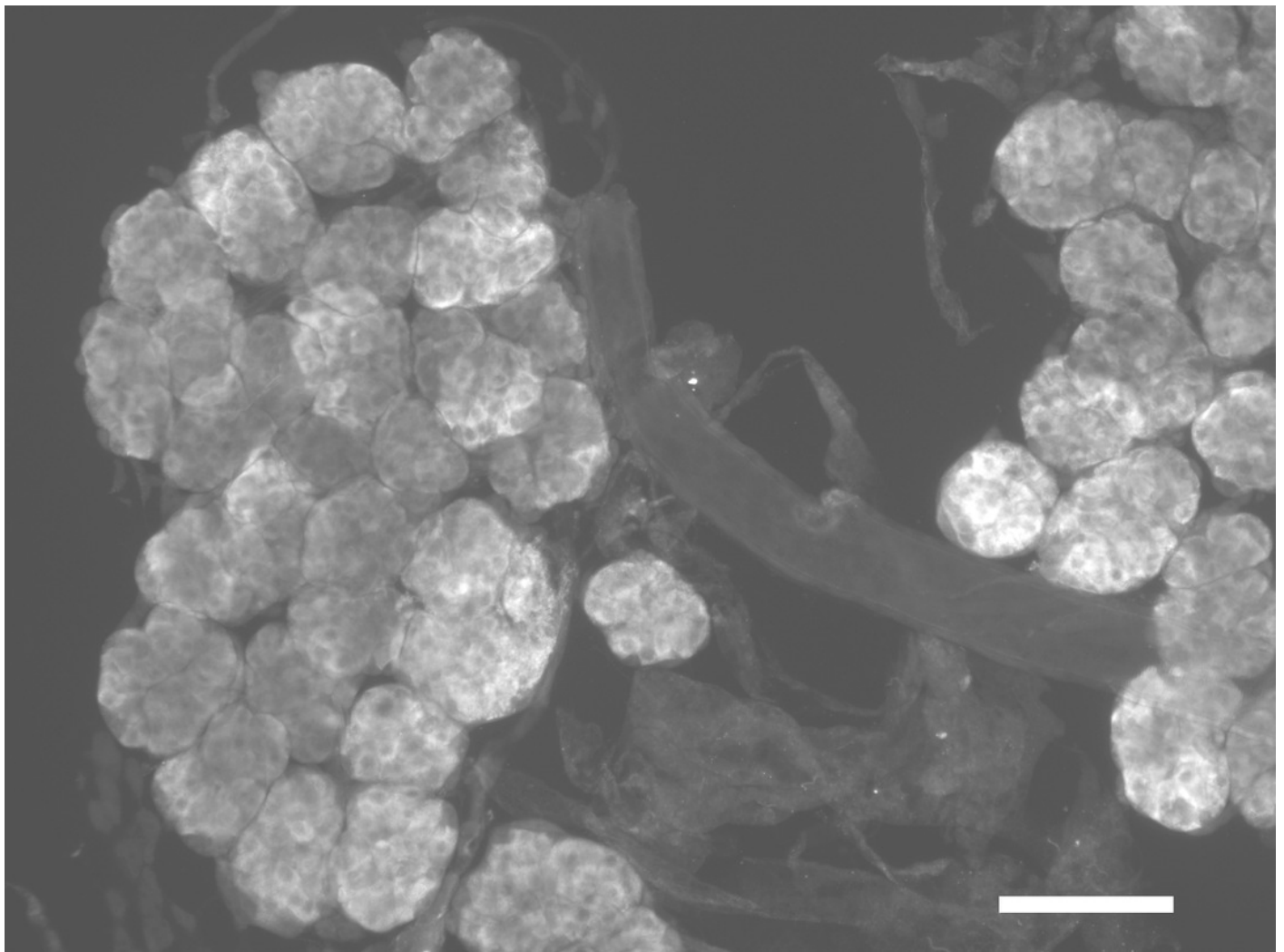


Figure 2

RT-PCR of the salivary gland peptide.

RT-PCR on different tissues from *L. migratoria* for the expression of SG-SASP and actin.

Numbers indicate the number of PCR cycles employed. Note that 20 cycles is sufficient to show expression in the salivary gland, while 35 cycles do not show any expression in the nervous system. TG 1 & 2, the pro- and meso-thoracic ganglion combined, TG3 & AGs the metathoracic ganglion together with all the abdominal ganglia. Complete gels with markers are shown in the supplementary data.

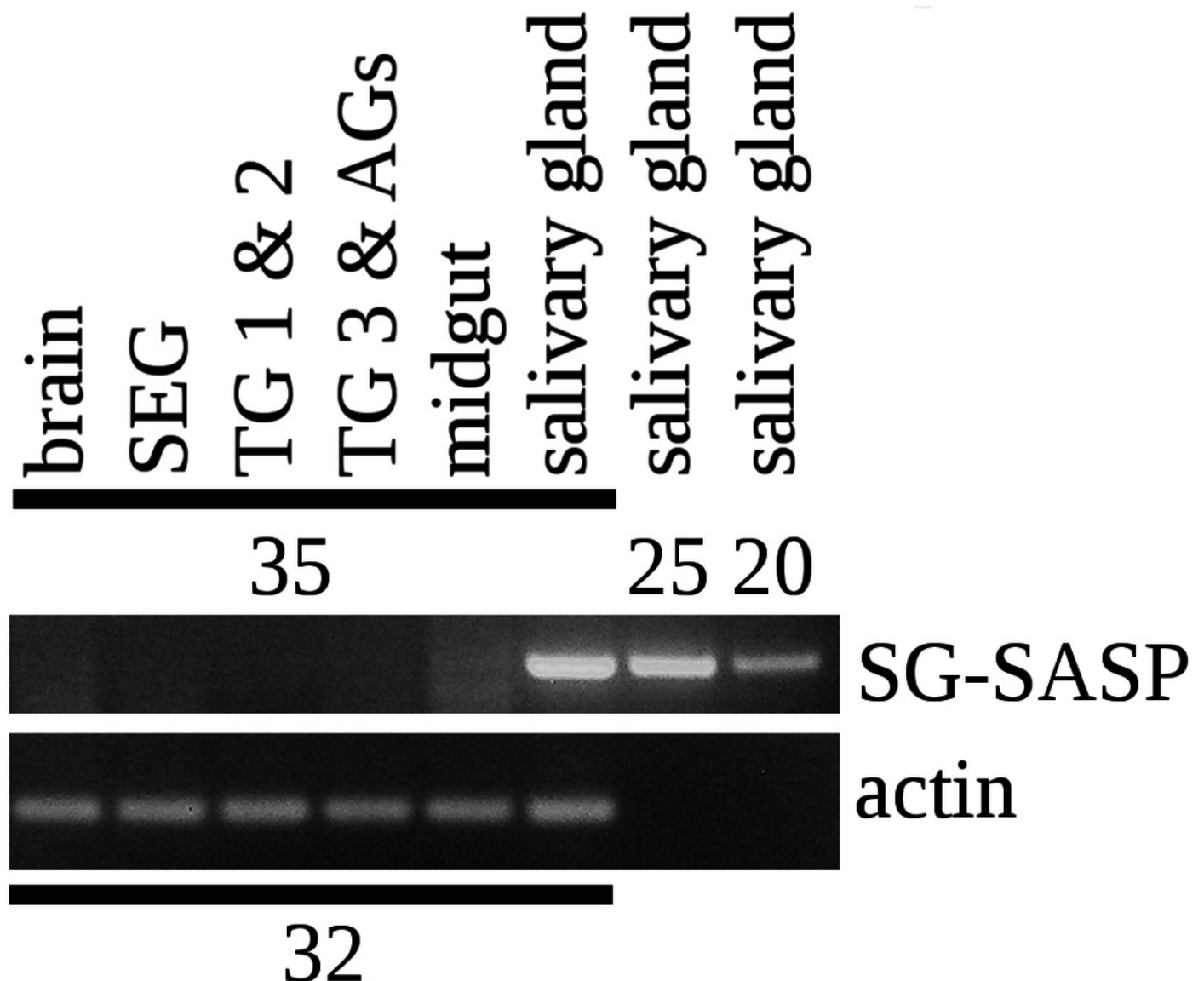
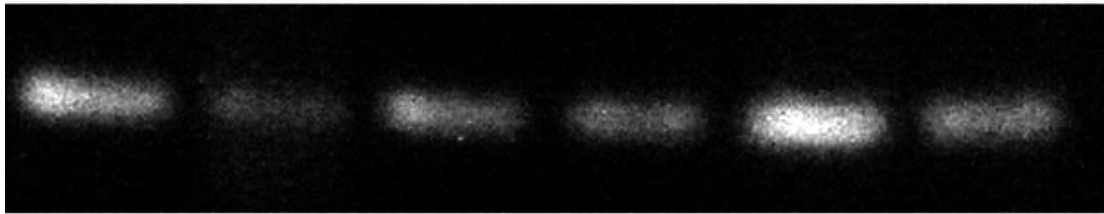


Figure 3

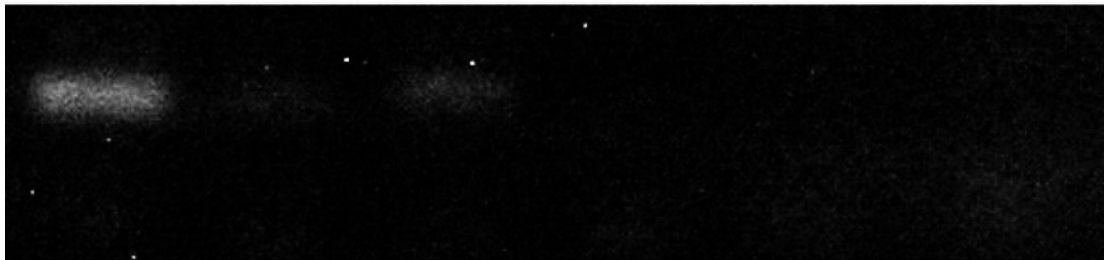
RT-PCR of PC1 and PC2.

RT-PCR on different tissues from *L. migratoria* for the expression of PC1 and PC2 convertases with actin as a control. Note that PC1 is widely expressed, including in the salivary gland, while the expression of PC2 is much more limited and not expressed in the salivary gland. Thirty two cycles of PCR in each sample. Complete gels with markers are shown in the supplementary data.

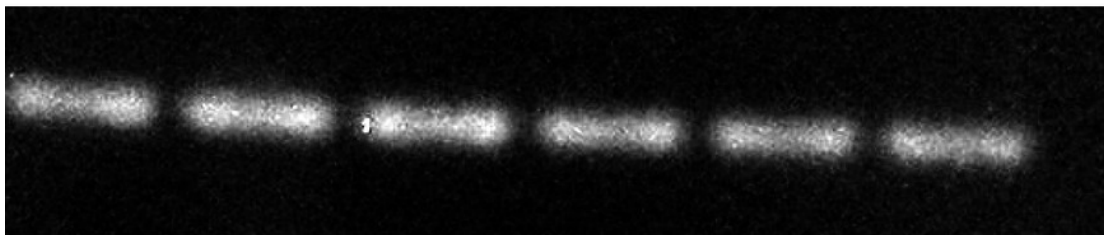
brain
midgut
Malpighian tubules
fat body
salivary gland
flight muscle



PC1



PC2



actin