Identification and characterization of pyrokinin and CAPA peptides, and corresponding GPCRs from spotted wing drosophila, *Drosophila suzukii*

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Abstract: The family of FXPRLamide peptides serves as a major insect hormone. It is characterized by a core active amino acid sequence conserved at the C-terminal ends, and provides various physiological roles across the Insecta. In this study we identified and characterized pyrokinin (PK) and Capa cDNAs encoding two FXPRLamide peptides, pyrokinin and CAPA-DH (diapause hormone), and two corresponding G-protein-coupled receptors (GPCRs) from spotted wing drosophila (SWD), *Drosophila suzukii*. Expressions of PK and CAPA mRNAs were differentially observed during all life stages except the embryo, and the detection of CAPA transcription was relatively strong compared with the PK gene in SWD. Both *D. suzukii* pyrokinin receptor (DrosuPKr) and CAPA-DH receptor (DrosuCAPA-DHr) were functionally expressed and confirmed through binding to PK and DH peptides. Differential expression of two GPCRs occurred during all life stages; a strong transcription of DrosuPKr was observed in the 3rd instar. DrosuCAPA-DHr was clearly expressed from the embryo to the larval stage, but not detected in the adult. Gene regulation during the life stages was not synchronized between ligand and receptor. For example, SWD CAPA mRNA has been up-regulated in the adult while CAPA-DHr was down-regulated. The difference could be from the CAPA mRNA translating multiple peptides including CAPA-DH and two CAPA-PVK (periviscerokinin) peptides to act on different receptors. Comparing the transcripts of SWD PK, CAPA, PKr and CAPA-DHr to four corresponding transcripts of *D. melanogaster*, SWD CAPA and the receptor are more similar to *D. melanogaster* than PK and the receptor. These data suggest that the CAPA gene could be evolutionally more conserved to have a common biological role in insects. In addition, the effect of Kozak sequences was investigated by the expression of the GPCRs with or without Kozak sequences in Sf9 insect cells. The Kozak sequenced PK receptor was significantly less active than the original (= no Kozak sequenced) receptor. Our results provide a knowledge for potential biological function(s) of PK and CAPA-DH peptides in SWD, and possibly offer a novel control method for this pest insect in the future.

Introduction

Neuropeptides are the largest group of insect hormones known to regulate a variety of physiological functions. The FXPRLamide neuropeptides are part of the PRXamide family based on the core active amino acids at the C-terminal ends with diverse functions across the Insecta [23, 24, 47]. The family of FXPRLamide peptides is defined in the C-terminal ends with the FXPRL (X is a variable amino acid; R or K in some cases) sequences, and amidated form (-NH₂). Various FXPRLamide neuropeptides identified are initially determined based on biological functions and amino acid homology, such as biosynthesis activating neuropeptide (PBAN), diapause hormone (DH), pyrokinin (PK), and capability (CAPA)-pyrokinin (CAPA-PK).

The first PBAN has been isolated (33-amino acids) from the subesophageal ganglion (SEG) to stimulate sex pheromone production in the corn earworm, *Helicoverpa zea* [48]. In the silkworm, *Bombyx mori*, similar peptides also have been identified from the heads to induce melanization and reddish coloration (MRC) on the larval epidermis, named MRC hormone (MRCH) [35]. In the same moth, DH was isolated (24-amino acids) from SEG complexes using a classical chromatography purification [21]. Later, it was determined that DH and PBAN, were translated from the same transcript in moths [26, 34, 51]. PK peptides belonging to the FXPRLamide family were isolated from cockroaches to stimulate the contraction of hindgut muscles [18, 43]. Most of CAPA mRNAs also translate PK peptides[45]. Insect CAPA genes are usually well conserved, and produce two family peptides, FXPRLamide (CAPA-pyrokinin, CAPA-PK) and PRVamide (CAPA-periviscerokinin (CAPA-PVK) at the C-terminal ends, respectively. The first CAPA-PK peptide has been identified from the abdominal perisymphatic organs (PSOs) of the cockroach [44].

Since the *Drosophila* genome was sequenced [1] a search for neuropeptides and receptors has been initiated and classified [17]. Two FXPRLamide peptides, PBAN-like (= PK-2) and DH-like (= PK-1) type peptides, were annotated from two different mRNAs in *D. melanogaster*, and characterized with a PBAN-immunoreactivity in the central nervous system (CNS) [8]. These two genes then have been identified, and these peptides were named as PK-2 and CAPA-3, respectively [27, 37].

Receptors of insect neuropeptides are particularly important to find signal transductions of neuropeptides that are involved with specific enzymes to produce biological products. Data mining and computational analysis from the *Drosophila* genome offered candidate genes for

potential receptors of FXPRLamide peptides [17, 53]. In particular, four G protein-coupled receptors (GPCRs) of *D. melanogaster* were annotated; their amino acid sequences were closely related to the vertebrate neuromedin U receptor (NmU-R) [17]. Using the similarity of the amino acid sequences conserved between NmU-R and *Drosophila* GPCRs, the first PBAN receptor has been identified from *H. zea* [6], followed by *B. mori* [20]. Two GPCRs annotated from the *Drosophila* genome were also discovered and characterized, functionally expressed, and determined receptors for PK-2 (= PBAN-like) and PK-1 (= DH-like) peptides [4, 49]. To date, more than a dozen GPCRs for PK-2 or PBAN-like have been identified, characterized and expressed from various insect groups [40]. However, PK-1 receptors (PK-1r or DHr) have been identified and characterized from only a few species, such as *B. mori* [19], *Anopheles gambiae* [41], *Rhodnius prolixus* [42]), *Aedes aegypti* [5] and *H zea* [22].

Spotted wing drosophila (SWD), *Drosophila suzukii*, is an economically damaging pest because the females have a serrated ovipositor, a unique morphological adaptation, enabling them to infest ripening fruit before harvest. Moreover, *D. suzukii* exhibits a wide host range in its introduced and native areas, infesting most small fruits including stone fruits [32]. SWD is an endemic pest in Asia [25], and recently invaded America [14, 15, 33, 50], and European countries [12]. The infestation area of SWD is rapidly expanding, and is now threatening the fruit industry. Control for *D. suzukii* has relied primarily on conventional insecticides although carrying many potential negative effects. The study of SWD recently has focused on development of sustainable managements and finding chemical insecticide alternatives such as biological and cultural methods [12].

In the present study we identified and characterized PK and CAPA cDNAs encoding FXPRLamide peptides, and confirmed two GPCRs for these peptides in SWD. Also, the differential expressions of the four mRNAs were characterized from the embryo to the adult. To determine if the Kozak sequences in the GPCR are necessary for the functional expression in the insect Sf9 cells, SWD receptors with or without the sequences were expressed and compared for their binding activities. Our results in this study provide basic knowledge to identify physiological function(s) of pyrokinin peptides and signal transduction which could be applied to the development of novel SWD control methods.

Materials and Methods

Flies

The *D. suzukii* used in these experiments has been in continuous colony in the Horticultural Crops Research Lab's insectary under L/D 16:8, RH 60±5% and 25±5 °C at USDA-ARS (Corvallis, OR, USA). Wildtype flies were collected in near the Corvallis area. Standardized rearing and hatching methods were applied for the SWD rearing described previously [57]. Adult males and females were collected daily for the examination of age related expression, as well as at various time points. Immature and adult fly samples were collected from eggs (~ 400 eggs), 1st instars (40 individuals), 2nd instars (30 individuals), 3rd instars (20 individuals), 1-day old pupae (30 individuals), 4-7 day old pupae (30), 1-day old adults (10 males and 10 females), and 7-day old adults (10 males and 10 females). Fly adult whole bodies and heads were also collected for RNA isolation, and then immediately processed to isolate RNA or frozen at -80°C.

RNA isolation and cDNA synthesis

Total RNA was isolated using the PureLink® total RNA mini Kit (Thermo Fisher Scientific, Waltham, MA, USA) from either fresh or frozen samples from the different developmental stages described above. Pooled samples were homogenized in cell lysis buffer using a microcentrifuge pestle and tube and tissue was macerated until no large pieces were visible. The isolation continued following the manufacturer's protocol and included a DNase digestion step to remove contaminating DNA. The isolated total RNA samples were analyzed on a Nanodrop 2000c (Thermo Fisher Scientific) to determine RNA concentration and immediately used for cDNA synthesis. Five micrograms (µg) of total RNA from each sample was converted into cDNA using SuperScript III First-Strand Synthesis System using oligo-dT as a primer according to the manufacturer's protocol (Thermo Fisher Scientific) and was then frozen at -20°C. The first-strand cDNA synthesized from the different tissues was used for cloning sequences and PCR amplification.

Cloning cDNAs for PK, CAPA, and GPCRs

The synthesized cDNA from each sample above was used to amplify the cDNAs of PK, CAPA, and two GPCRs. The sequence homologs found in *D. melanogaster* are utilized to amplify the cDNAs from the published *D. suzukii* genome (<http://spottedwingflybase.oregonstate.edu/>).

A partial sequence of *D. suzukii* PK DNA was amplified with a specific primer set: sense

primer, 5'-ATGTGTGGTCCCAGTTATTGCACAC-3' and antisense primer, 5'-GCACCGATTCTTGGCCATG-3' based on the nucleotide sequence of the spotted wing flybase data. PCR was performed with the following temperature program: 35 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min. The PCR product (505 nucleotides) was gel purified and cloned using a PCR cloning vector pSC-A-amp/kan per manufacturer's instructions (Agilent Technologies, La Jolla, CA, USA), and sequenced by Center for Genome Research and Biocomputing (CGRB), Oregon State University (Corvallis, OR, USA). Based on the DNA sequence result a forward primer, 5'-ATTGAGTGAAGGGCGCCAAA-3', was designed to use 3'RACE (Rapid Amplification of cDNA Ends) using a 3' RACE kit (Thermo Fisher Scientific). Then, a primer set, sense primer, 5'-ATGTGTGGTCCCAGTTATTGCACAC-3' and antisense primer, 5'-TCAATCGCCGCCGCACACTTG-3', to amplify a full-length of the cDNA. The obtained full-length (555 nucleotides) sequence information was aligned and sequences compared with other known pyrokinin sequences using Geneious® DNA software (ver 8.1.5).

The full length of clone the *D. suzukii* CAPA DNA was amplified with a specific primer set: sense primer, 5'-ATGAAATCTATGTTGGTCCAC-3' and antisense primer, 5'-CTAGTTGAG TTCCTTCTGTCC-3' based on the nucleotide sequence retrieved from the SpottedWingFlyBase. PCR was performed with the following temperature program: 35 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min. The obtained full-length (456 nucleotides) sequence information was compared with other known CAPA sequences using the same method above.

A putative sequence of *D. suzukii* PK receptor was found in the SWD genome above by BLAST search using the known *D. melanogaster* PK-2 receptor (CG8795, Accession No. NP_731788) [49]. The sequence included the start and stop codons, but much longer (2,190-bp) than other *Drosophila* PK-2 receptors (~1,780-bp). A primer set: sense primer, 5'-ATGCTGCCCAGCAACAGTTCCGGT-3' and anti-sense primer, 5'-TTATTAAAAAGCCGATCCCCGCTCCC-3' were designed to amplify a full-length of the *D. suzukii* receptor under a PCR condition of 35 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 2 min. The PCR products were purified, sequenced and analyzed by the same method and procedure described above.

A putative *D. suzukii* CAPA-DH-1r (= CAPA-PK-1r) was also found by BLAST search using the known DH receptor from *D. melanogaster*'s GPCR (= CG9918, Accession No, NP_001014620) [4]. The full-length of *D. suzukii* CAPA-PK-1r was amplified using the specific

primer set: sense primer, 5'-TTATGTCCGCTGGCAATGTGAGCC-3' and anti-sense primer, 5'-TCATCAGTTGACTTGTTTCGTTGATCA-3' under similar PCR conditions as above. The PCR product was cloned, sequenced and analyzed by the same method and procedure described above.

Functional expression of GPCRs and binding assay with peptides

The open reading frame (ORF) of *D. suzukii* PK receptor was inserted into a pIB/V5-His TOPO® TA expression vector (Thermo Fisher Scientific) for expression in Sf9 cells as described previously [6]. To add a Kozak sequence (**G/ANNATGG**) in the translation initiation the ORF of *D. suzukii* PK receptor was amplified using a primer set: sense primer, 5'-ATGGTGCCCAGCAACAGTTCCGGT-3' and the same antisense primer above. The sequences of two different ORFs of *D. suzukii* PK receptor were confirmed after insert into the expression vector. The ORF of *D. suzukii* CAPA-PK-1r was amplified using the sense primer, 5'-TTATGTCCGCTGGCAATGTGAGCC -3' and the anti-sense primer, 5'-TCATCAGTTGACTTGTTTCGTTGATCA-3'. The PCR product was inserted into the same vector for functional expression as described above.

The two peptide ligands (PK and Cap2b-3 from *D. suzukii*) used in this study were synthesized or purchased from AnaSpec, Inc (Fremont, CA, USA). The preparation of cells, peptides, and Fluo-4AM (Thermo Fisher Scientific) with a lepidopteran saline followed or modified a previously described method [6]. The receptor transfections in Sf9 cells were performed to select stably transfected cells by blasticidin. Cell fluorescence intensity in a 96-well cell-culture plate was measured using a FlexStation 3 multi-mode microplate reader (Molecular Devices, Sunnyvale, CA, USA) equipped with filters (excitation: 485 nm and emission: 520 nm), and an 8-channel pipettor. Fluorescence measurements from each well on the column were taken every 5s. After 30s the peptide ligand (10 µL) was added by pipettor and changes of fluorescent intensity were measured for up to 3 min. Then, 5 µL of 1 µM ionomycin (Thermo Fisher Scientific) was added to the cells to obtain a maximum fluorescence reading. The effect of ligand-exposure was expressed relative to the maximum value obtained with ionomycin. Data were analyzed using Microsoft Excel as described previously [6] and measured EC₅₀ values of ligands were determined using GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA, USA). The transmembrane domains (TMs) was predicted using a 3D topology program, CCTOP server (<http://cctop.enzim.ttk.mta.hu/>)

Reverse transcriptase (RT)-PCR for cDNAs of PK, CAPA-DH, PKr and CAPA-DHr

To investigate gene expression, total RNA (5µg) isolated from each life stage was synthesized to cDNA as described above. One µL of cDNA (20 µL total) from each stage was used to amplify for partial lengths of four *D. suzukii* cDNAs: PK (350-bp) with a primer set (5'-ATTGAGTGAAGGGCGCCAAA -3' and 5'-GCACCGATTCTTGGCCATG -3'), CAPA-DH (350-bp) with a primer set (5'-GGTGCCAACATGGGTCTCTA-3' and 5'-TGTCCTTGTGTTGGTGTCTC-3'), PKr (337-bp) with a primer set (5'-ACAACCTTCATGCACACGGC-3' and 5'-ATTGGATGGCTTGGGGAAGG-3') and CAPA-DHr (426-bp) with a primer set (5'-AGGAGGTGGCCTTCATTTGG-3' and 5'-CAGGACCCTCCACCAAAGTC-3'). PCR for each target was performed as follows: 94 °C for 3 min, and 35 cycles at 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 30 s, then 72 °C for 5 min with *i*Taq polymerase (Bio-Rad, Hercules, CA, USA). A 254-bp fragment of the *D. suzukii* Snf7 mRNA (a housekeeping gene) was also amplified as a positive control using primers (5'-CGCGAGACGGAGAACATGCT-3' and 5'-TCCGCGGCATTCTTCATCGT-3'). Then, each PCR product was checked for amplification using 1.2% agarose gel electrophoresis and visualized using GelRed™ (Biotium, Hayward, CA, USA) under a UV light. The expected PCR products were purified and cloned, then confirmed by DNA sequence (CGRB, Oregon State University).

Results

PK (=Hugin) and CAPA mRNAs and pre-propeptides

Drosophila suzukii PK (DrosuPK) mRNA (GenBank Accession No. KX768139) encoded a 184-amino acid (AA) protein possessing two FXPRL peptide homologs (underlined Fig. 1) by potential endoproteolytic cleavage sites (underlined with italics in Fig. 1A). The cleaved peptides are predicted to have a C-terminal amide group provided by glycine (G) [52, 54]. There are considered two peptide homologs, 17-AA (QLQSNGEPAYRVRTPRLamide) for hug gamma (hug γ), and 8-AA (SVPFKPRLamide) for PK. Both homologs are identical with the sequences found in *D. melanogaster* [8, 37]. But, the sequence for the hug γ in the previous report of *D. suzukii* [2] is slightly different in this study. The transcription of SWD PK mRNA was investigated in all developmental stages (Fig. 1B). The mRNA was expressed in the larvae,

pupae and adults, but not in the egg. As expected the ORF amino acid sequences of the SWD PK cDNA were conserved with ~ 85% similarity compared to *D. melanogaster*. The length of the SWD PK was 7-AA shorter than *D. melanogaster* although sequences of hug γ and PK peptides were not different between the two species.

Drosophila suzukii CAPA (DrosuCAPA) mRNA (GenBank Accession No. KX768140) encoded a 151-AA protein possessing three putative peptides (underlined) by potential endoproteolytic cleavage sites (italics in Fig. 2A). These are considered two CAPA-periviscerokinins (CAPA-PVKs), 12-AA (GANMGLYAFPRVamide) and 10-AA (ASGLVAFPRVamide), and one CAPA-DH, 15-AA (TGPSASSGLWFGPRLamide). Recently, the presence of three peptides has also been confirmed in *D. suzukii* by a mass spectrometric analysis [2], and found with same sequences from *D. melanogaster* [8, 27]. The expression of the CAPA mRNA was also investigated in all developmental stages and adult (Fig. 2B). The gene transcription was obviously detected from the post-embryo stages, and seems increased to the pupae and adults. The AA sequences encoded from CAPA cDNAs were ~91% identical with the same ORF length between *D. suzukii* and *D. melanogaster*, indicates CAPA gene could be more conserved than PK gene in two species.

Identification of GPCRs for PK and CAPA-DH

Two putative GPCRs for SWD PK and CAPA-DH were amplified using a PCR-based cloning strategy. The amino acid sequence (585-AA, GenBank Accession No. KX768141) was then identified and confirmed as the *Drosophila suzukii* pyrokinin receptor (DrosuPKr) through a combination of functional expression and a peptide binding assay. The amino acid sequences from the N-terminal side to the 7th transmembrane from DrosuPKr is almost identical, with 90 % identities overall, with *D. melanogaster* PKr. But, the intracellular C-terminal sequences are less homologous and reduced compared with *D. melanogaster* (Fig. 3A). The expression of DrosuPKr was investigated using reverse transcriptase (RT) PCR in various SWD life stages. The PKr was strongly expressed in the 3rd instar larvae, but very weak or not detectable in the other life stages (Fig. 3B), which is interesting that the functional activity of PKr is stage specific.

The full sequence (429-AA, GenBank Accession No. KX768142) for *Drosophila suzukii* CAPA-DH receptor (DrosuCAPA-DHr) was identified and confirmed through also functional expression and peptide binding assay. The amino acid sequence similarity between *D. suzukii*

and *D. melanogaster* showed 92 % identities, and little different in the C-terminal parts (Fig 4A). The expression of DrosuCAPA-DHr was clearly observed in the egg and all larval periods, and not in pupae and the adults (Fig 4B), and showed very different expression mode to DrosuPKr (Fig. 3B).

Receptor binding activity with peptides

SWD receptors were functionally expressed and simply maintained in Sf9 cells as described previously [6]. A calcium assay method was utilized to determine peptide binding activity by half-maximal effective concentration (EC_{50}) values using a fluorescence plate reader to detect changes of fluorescence intensity. The same family of receptors from *D. melanogaster* were also cloned, expressed and compared by peptide binding activity. *Drosophila* PK receptors were well activated by PK peptide but not by CAPA-DH (Table 1 & Fig. 5). For example, EC_{50} values of PKrs to PK peptide (SVPFKPRLamide) had 20 nM in *D. suzukii* (DrosuPKr) and 70 nM in *D. melanogaster* (DromePKr), respectively, but over 5 μ M to CAPA-DH peptide (TGPSASSGLWFGPRLamide). In contrast, EC_{50} values of CAPA-DH receptors to CAPA-DH had 16 nM from DrosuCAPA-DHr, and 12 nM from DromeCAPA-DHr, respectively, but over 5 μ M with PK peptide from both receptors (Table 1).

To determine the effect of Kozak sequences (bold letters), **G/ANNATGG**, on translation initiation and an ATG start codon for GPCR expression [29, 30] the forward PCR primer of SWD PKr was designed with or without Kozak sequences. Two DrosuPKrs were transfected and expressed in Sf9 cell, and measured binding affinities to PK peptide (Fig. 5). The binding activity of DrosuPKr without Kozak sequences was stronger than the modified DrosuPKr with Kozak sequence in the start codon. The result indicates, Kozak sequence in insect GPCRs might be not necessary, even interfering the receptor binding activity. However, vertebrate GPCRs might need the sequences to initiate properly the translation in cells.

Discussion

PK, PBAN, and DH peptides belonging to FXPRLamide family are sometimes confused due to different names from a variety of structural and functional diversities found in many insect groups. In general, FXPRLamide peptides are produced from two different genomic sources, PK/PBAN/DH and CAPA genes. In most of the insect Orders thus far, PK/PBAN/DH mRNAs produce three to five FXPRLamide [10], while *Drosophilidae* flies are expected to translate two

peptides known as hug γ and PK (= PK-2 type) peptides [8, 37]. Insect CAPA mRNAs usually encode a single FXPRLamide, CAPA-DH (= PK-1 type) peptide, and several PRVamide peptides, CAPA-PVKs [13, 45]. To date, hundreds of FXPRLamide family peptides found over 150 species are well conserved across Insecta [reviewed by 23], although their physiological functions are unclear in many parts.

One of two FXPRLamide homologs from SWD PK transcript, called hug γ (QLQSNGEPAYRVRTPLamide), is ambiguous with the amino sequence and the production as a mature protein. A similar sequence (EQNVQSNGEPAYRVRTPLamide) has been proposed for SWD hug γ [2], which is slightly different (underlined) in the N-terminal part from the present study. In this study, SWD hug γ is also completely identical with the amino acid sequences encoded from *D. melanogaster* hug in mRNA [37]. So, the sequencing difference could be from an annotation error in the SWD genome. Although the amino acid sequences for hug γ peptide are common in Diptera including flies and mosquitoes [10], there is an ambiguity for the presence as a mature peptide produced in the cell because this peptide was never detected or isolated by peptidomic analysis yet [3, 56]. In SWD the presence of a PK peptide (SVPFKPRLamide) was confirmed using a mass spectral analysis, but the hug γ was not detected [2]. In addition, the mono basic residue (R) in the N-terminus could not provide a cleavage site to process a functional peptide. This needs to be confirmed by another method in the future.

SWD PK mRNA is differentially expressed during postembryonic development, but barely detectable in the egg. Similar findings also occurred during all life stages in *D. melanogaster* [37; unpublished data by Y.H.K]. The immunocytochemistry for FXPRLamide peptides using the anti-sera in the central nervous system (CNS) of *D. melanogaster*, demonstrated that the peptide is produced and released from the larva to adult [8]. In another insect species, the similar pattern of the PK gene expression and/or the peptide immunocytochemistry are found in the fire ant [9, 11], mosquitoes [5, 16] and sand fly [10]. Although the immunolocalization of FXPRLamide peptides from these insects was detected in the brain, subesophageal ganglion (SEG), thoracic ganglion (TG) and abdominal ganglion (AG) the PK mRNA might not be expressed in the AG, because it was not amplified in our previous studies [5, 10, 11]. The immunoreactivity of FXPRLamide peptides across the central nerve tissues could be resulted from the mixed presence with multiple FXPRLamide peptides encoded from PK or CAPA mRNAs.

Since the first CAPA-DH (= PK-1 type, not CAPA-PVKs) peptide was isolated from the cockroach [44] a similar peptide for *Drosophila* CAPA-DH has been proposed and/or isolated using proteomics and genomics tools [8, 27, 46, 56]. The immunocytochemical localization of CAPA peptides in *Drosophila* species showed 3 pairs of neurons in the AG and 1 pair of neurons in the SEG [27, 45]. Although FXPRLamide anti-sera can recognize specific CAPA-DH (TGPSASSGLWFGPRLamide) from the CAPA gene in *Drosophila* the distribution of immune-stained neurons could contain another FXPRLamide peptide, PK (SVPFKPRLamide) from the pyrokinin gene. To avoid potential cross-immunoreactivity it is a better way to investigate CAPA mRNA expression in the CNS. The transcription of CAPA mRNA was clearly detected in all CNS tissues, brain, SEG, TG, and AG, in the fire ant [7]. CAPA peptides are actively produced in the CNS and not the other tissues in insects [45]. SWD CAPA mRNA was also strongly expressed in all life stages except egg barely detected. Similar results were found in *D. melanogaster* (data not shown by Y.H.K.), and the fire ant PK mRNA was also expressed during all life stages [7].

Physiological roles of PK peptides (PK -1 & 2) in *Drosophila* or other Dipteran species are not fully understood yet, but could be implicated in the control of hyperneural muscle contraction, accelerate pupariation, food intake, growth and metabolism [36-38, 45, 55, 58]. To identify biological functions for FXPRLamide peptides it is important to study their receptors.

In this study, the amino acid sequence from each GPCR is very similar to the corresponding GPCR identified from *D. melanogaster* [4, 49]. PK receptors (PKrs) have a long extracellular C-terminal end compared with CAPA-DHrs in *Drosophila*. Unlike two peptide ligands (PK and CAPA), their receptors are expressed in developmental stages other than the adult, and mostly detected in the larval stage. In particular, a strong signal for DrosuPKr transcription was observed only in the 3rd instar, whereas DrosuCAPA-DHr is obviously detected during the larval and embryonic stages as reported in the mosquito [5] and fire ant [7]. But, none or barely any expression for two receptors were detected in the pupae and adult periods when PK and CAPA mRNAs are being upregulated. The disparate expressions between ligands and their receptors could be resulted that PK and CAPA mRNAs produce multiple peptides activating different receptors. For example, DrosuCAPA mRNA produces not only CAPA-DH but also two CAPA-PVK peptides to have their own receptors activated during different stages. Insect PBAN and PBAN receptor genes are up- or down-regulated depending on life stages and species [5, 31, 40,

59]. It could be also sometimes resulted by different detection methods or species-specificity. The binding strength measured by EC₅₀ value (20nM) for DrosuPKr to PK is three times higher than *D. melanogaster* (70nM), but it is not significant (16nM vs. 12nM) at DrosuCAPA-DHr to CAPA-DH in two species. Apparently, the binding activities of two receptors to PK and CAPA-DH are controversial, that PKr is only activated by PK not CAPA-DH, and vice versa in CAPA-DHr, indicating that receptors for PK1 (= DH type) and PK2 (= PBAN type) are clarified in insects.

To express membrane-bound proteins including 7-transmembrane proteins (= GPCRs) in eukaryotic cells Kozak sequences (**G/ANNATGG**) have been recommended or required for the translation initiation [29, 30]. Currently, use of a commercially available expression vector such as pIB/V5-His TOPO® TA expression vector (Thermo Fisher Scientific) recommends to insert Kozak sequences for the translation initiation and an ATG start codon, even unsure to be applied in insect cells. Sf9 insect cells expressing SWD PK receptors with or without Kozak sequences produces GPCRs to be localized in the cell membrane. However, the Kozak sequenced DrosuPKr binding to PK peptide is significantly less active than the original (= no Kozak sequenced) DrosuPKr. This means the Kozak sequences in insect GPCRs for the translation initiation in Sf9 cells are not recommended, and it could interfere the translation and/or the binding activity between receptor and peptide.

Overall, comparisons of amino acid sequences from PK, CAPA, PKr and CAPA-DHr cDNAs between *D. suzukii* and *D. melanogaster* SWD genes are relatively simplified and reduced. From the molecular evidences SWD could be recently evolved species shifting to ripen and fresh fruits to avoid a food competition with other *Drosophila* species being attracted to decayed fruits [28]. Recently, our study demonstrated the more reduced gene numbers and activity of glutathione-S transferase (GST) in SWD also suggested the evolutionary adaptation to fresh foods by alterations of the GST [39]. As compared all four transcripts in *D. suzukii* and *D. melanogaster*, CAPA and CAPA-DHr cDNAs have more homology to corresponding cDNAs between two species than PK and PKr, suggests that CAPA and its receptor might be more conserved in the evolutionary process. In fact, CAPA genes found are ubiquitous across Invertebrate phyla including Arthropoda [45]. This study provides a foundation to find the function(s) of PK and CAPA-DH peptides, and to develop a novel strategy for SWD control.

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Table 1. Half-maximum effective concentrations (EC_{50}) of the peptides tested on the expressed *D. melanogaster* and *D. suzukii* pyrokinin (PK) and CAPA-DH receptors

Peptide	Sequence	DrosuPKr	DromePKr	DrosuCAPA-DHr	DromeCAPA-DHr
Pyrokinin (PK)	SVPFKPRLa*	150 nM	120 nM	> 5 μM	> 5 μM
CAPA-DH (Cap2b-3)	TGPSASSGLWFGPRLa	> 5 μM	> 5 μM	196 nM	150 nM

EC₅₀ values were determined by using log-probit analysis with 4 replicates from each peptides. Maximum fluorescence readings were obtained by treating cells with ionomycin after challenge with peptides. *amide in the C-terminal end. Replicated four times at least for each test.

Figure Legends

Figure 1. A: Alignment of amino acid sequences encoded from PK mRNAs of *D. suzukii* and *D. melanogaster*, and putative FXPRLamide peptide homologs (underlined). Potential endoproteolytic cleavage sites are in italics. Amino acid sequence homologs are hug γ (QLQSNGEPAYRVRTPL) and PK (SVPFKPRL), respectively. **B:** Expression of DrosuPK mRNA from egg (E), 1st instar (L1), 2nd instar (L2), 3rd instar (L3), 1- & 4- day old pupae (P1, P4), and 3- & 7-day old adults (A3, A7). A 254-bp fragment of the SWD Snf7 mRNA was amplified as a positive control. GenBank accession No: DrosuPK (KX768139) and DromePK (NP_524329).

Figure 2. A: Alignment of amino acid sequences encoded from *D. suzukii* and *D. melanogaster* CAPA mRNAs, and putative peptides (underlined): CAPA-PVK1(GANMGLYAFPRV), CAPA-PVK2 (ASGLVAFPRV) and CAPA-DH (TGPSASSGLWFGPRL). Potential endoproteolytic cleavage sites are in italics. **B:** Expression of DrosuCAPA mRNA from egg (E), 1st instar (L1), 2nd instar (L2), 3rd instar (L3), 1- & 4- day old pupae (P1, P4), and 3- & 7-day old adults (A3, A7). A 254-bp fragment of the SWD Snf7 mRNA was amplified as a positive control. GenBank accession No: DrosuCAPA (KX768140) and DromePK (NP_524552).

Figure 3. A: Alignment of amino acid sequences encoded from PKrs of *D. suzukii* and *D. melanogaster*, and 7-tranmembranes (underlined). **B:** Expression of DrosuPKr mRNA from egg (E), 1st instar (L1), 2nd instar (L2), 3rd instar (L3), 1- & 4- day old pupae (P1, P4), and 3- & 7-day old adults (A3, A7). A 254-bp fragment of the SWD Snf7 mRNA was amplified as a positive control. GenBank accession No: DrosuPKr (KX768141) and DromePKr (NP_731788).

Figure 4. A: Alignment of amino acid sequences encoded from CAPA-DHrs of *D. suzukii* and *D. melanogaster*, and 7-tranmembranes (underlined). **B:** Expression of DrosuCAPA-DHr from egg (E), 1st instar (L1), 2nd instar (L2), 3rd instar (L3), 1- & 4- day old pupae (P1, P4), and 3- & 7-day old adults (A3, A7). GenBank accession No: DrosuCAPA-DHr (KX768142) and DromeCAPA-DHr (NP_001014620).

Figure 5. Dose–response profiles of the effect of synthetic DrosuPK peptide (SVPFKPRLamide) on *D. suzukii* PK receptor contained Kozak sequence (G/ANNATGG) or without Kozak sequence. *D. suzukii* PK receptors were expressed in Sf9 cells. Maximum fluorescence readings were obtained by treating cells with ionomycin after challenge with peptides. The y-axis indicates the peak peptide fluorescence value relative to ionomycin treatment (mean $\pm$ 4 s.e.m).

A

DrosuPK MCGPSYCTLLIIAASCYVLVCSQAKSLQGT SKQDLGNHVS GGAARGSS--SSPTLSEGRQ
 DromePK MCGPSYCTLLIIAASCYILVCSHAKSLQGT SKLDLGNHISAGSARGSLSPASPALSEARQ
 *****;****;***** *****;*. *;***** ;*:***. **

DrosuPK KRAMGEYKELTD I IDELE ENSLAQKASATL FVGAVP PQGQEF DLD SMP PLTY YLL LQKLR
 DromePK KRAMGDYKELTD I IDELE ENSLAQKASATM QVAAMP PQGQEF DLD T MP PLTY YLL LQKLR
 *****;*****;*****: *. *;*****;*****

DrosuPK QLQSNGE PAYRVRI PRLGRSIDSWRILDGEGA-----PEESI GGQFVQRM AKKSVFFKPR
 DromePK QLQSNGE PAYRVRI PRLGRSIDSWRLDAE GATGMAGGEEA IGGQFMQRM VKKSVFFKPR
 *****;***.*** **;*****;***.*****

DrosuPK LGKRAQVCGGD 184
 DromePK LGKRAQVCGGD 191
 *****

B

A

```
DrosuCAPA      MKSMLVNAVLVFLIAEFSAAEEMDHDKNRRGANMGLYAFPRVGRGDFSLANSLRDGLAAG
DromeCAPA      MKSMLVHIVLVIFIIAEFSTAE TDHDKNRRGANMGLYAFPRVGRGDFSLANSLRDGLAAG
*****. **.;*****;*****.*****

DrosuCAPA      FLDGIYGDASQEDYNEADFQKRSGLVAFPRVGRGDAELRKWAHLLALQQVLDKRTGPSA
DromeCAPA      VLDGIYGDASQEDYNEADFQKRSGLVAFPRVGRGDAELRKWAHLLALQQVLDKRTGPSA
*****.*****

DrosuCAPA      SSGLWFGPRLGKRSVDAKSPEDINKGQKEFN      151
DromeCAPA      SSGLWFGPRLGKRSVDAKSFADISKQKELN      151
*****.*****;
```

B

B

	E	L1	L2	L3	P1	P4	A3	A7	
DrosuCAPA									350-bp
Snf7									254-bp

RT-PCR analysis of DrosuCAPA and Snf7 expression. The gel shows bands for DrosuCAPA (350-bp) and Snf7 (254-bp) across eight lanes: E (empty vector), L1, L2, L3 (LacZ reporter), P1, P4 (P-element reporter), A3, and A7 (A-element reporter). DrosuCAPA expression is induced by the LacZ and P-element reporters but not by the A-element reporter. Snf7 expression is consistent across all lanes, serving as a loading control.

A

DrosuPKr	MLPSN SSGLLATDLQLFHNEKFLNLNTQVLNISADNLTSLQGLEPEELLPTATPMTPLS	
DromePKr	MLPTN SSGVLATDLQLFHNEKFLNLNTQVLNISADNLTSLQGLEPEELLPTVTPTMTPLS	
	::*****.*****	
DrosuPKr	LLATLSVGYALIFIAGVLGNLITCIVISRNNFMHTATNFYLFNLAISDMILLCSGMPQDL	
DromePKr	LLATLSVGYALIFIAGVLGNLITCIVISRNNFMHTATNFYLFNLAISDMILLCSGMPQDL	
	*****	
DrosuPKr	YNLWHPDNYPFSDSICILESVLSEAAANATVLTITAFIVERYIAICH PFRQHTMSKLSRA	
DromePKr	YNLWHPDNYPFSDSICILESVLSEAAANATVLTITAFIVERYIAICH PFRQHTMSKLSRA	
	*****	
DrosuPKr	VKFIFAIWIAALLLALPQAIQFSVVMQGMGSSCTMKNDFFAHVFAVSGFLFFGGPMTAIC	
DromePKr	VKFIFAIWIAALLLALPQAIQFSVVMQGMGTSCITMKNDFFAHVFAVSGFLFFGGPMTAIC	
	*****	
DrosuPKr	VLYVLIGVKLKRSRLQLALPRRCYDVRNGISAQTRVIRMLVAVAVAF FICWAPFHAQRLM	
DromePKr	VLYVLIGVKLKRSRLQLALPRRCYDVRNGISAQTRVIRMLVAVAVAF FICWAPFHAQRLM	
	*****	
DrosuPKr	AVYGSTSGIESQWFNDVFSILDYTSGRPLPLHCINPLEYINIMSHKFREAFKVTILARQFG	
DromePKr	AVYGSTSGIESQWFNDVFSILDYTSGLVYFLSTCINPLLYNIMSHKFREAFKVTILARHFG	
	***** : *****	
DrosuPKr	LGGMNQGRGLPHTYSALRRNQTSRLRLHTDSVRTIMTSTTTTTGLNGCGNGS--GAAT	
DromePKr	LGGMNQGRGLPHTYSALRRNQTSRLRLHTDSVRTIMTSMATTTTGLNGSANGSGNGTTT	
	***** : *****	
DrosuPKr	GPAVRLNRVSLDSSQLQGQNRSRQDLFDNPRRLQTQISQLSSVGDASHLLEE DLQFPGE	
DromePKr	GQSVRLNRVSLDSVQMGGQNRSRQDLFDNPRRLQTQISQLSSVGDASHLLEE DLQFPGE	
	* :*****	
DrosuPKr	PLQRQPTMCSDILTDOLAISRSLKLITRAPLAFVGAGG-----VAGGVGGDES	
DromePKr	PLQRQPTMCSDILTDOLAISRSLKLITRAPPGVTVGGVAGGSTTGAAGSGGVGGDES	
	***** . * . *	
DrosuPKr	SGKYRKAKVKALKSSSALKGLRAKFNWRARRKGSHKPKVKEATGAVDDAGERGSAF	585
DromePKr	SGKYRKAKVKVLKSSSPFKGLATKFNWRARRKGSHKPKHEKATVNGGDTEERAAF-	595
	***** :*****	

B

[illegible]

A

DrosuCAPA-DHr	MSAGNVSHVLGPPRDPLAIVIPVTWQYSLIFISGVVGNISTCIVIMGNRSMHTATNYLFF
DromeCAPA-DHr	MSAGNMSHDLGPPRDPLAIVIPVTWVYSLIFITGVVGNISTCIVIMGNRSMHTATNYLFF
	*****; ** *****;*****;*****
DrosuCAPA-DHr	SLAISDFLLLLSGVPOEVAFIWSKYPIYFGEYICIGBGLLAETSANATVLIITAFTVERY
DromeCAPA-DHr	SLAISDFLLLLSGVPQEVSYIWSKYPIYFGEYICIGBGLLAETSANATVLIITAFTVERY
	*****;*****;*****;*****
DrosuCAPA-DHr	IAICHPFLGQAMSKLSRAIRIIVLVNIMAVVTAIPQAAQFGIEFYSGVEQCTIVRVIVKH
DromeCAPA-DHr	IAICHPFLGQAMSKLSRAIRIIVLVNIMAVVTAIPQAAQFGIEHYSGVEQCGIVRVIVKH
	*****;*****;*****
DrosuCAPA-DHr	SFQLSTFIFFLAPMSIILVLYLLIGVHLYRSTLVEGPASVARRQQLKSVPSDTIILYRYAG
DromeCAPA-DHr	SFQLSTFIFFLAPMSIILVLYLLIGVHLYRSTLVEGPASVARRQQLKSVPSDTIILYRYGG
	*****;*****;*****
DrosuCAPA-DHr	SSTAVNHLNGGGGGGA-AAVGGSGAQLSSVRGRNLNHYGTRRVLRLMLVAVVVCFFLCWAPF
DromeCAPA-DHr	SGTAMSFNGGGSGAGTAGLMGGSGAQLSSVRGRNLNHYGTRRVLRLMLVAVVVCFFLCWAPF
	*.**:.;****.**: . :*****;*****
DrosuCAPA-DHr	HAQRLLAIYAPARGAKLREQHEFVYTVMTYISGVLYYLSTCINPLLYNIMSHKFREAFKA
DromeCAPA-DHr	HAQRLLAIYAPARGAKLRDQHEFVYTVMTYVSGVLYYLSTCINPLLYNIMSHKFREAFKA
	*****;*****;*****
DrosuCAPA-DHr	VLFGKKASKGSLTSRTNMSRRLRRALTNS SQTQRVSIESAEQPKQFVMQNFVNRPTVAS
DromeCAPA-DHr	VLFGKKVSKGSLNSRNIE SRRLRRALTNS SQTQRFSIESAEQPKFSIMQNFPTNKPFVAA
	*****;*****;**.**:*****;*****;*****;****.**:**
DrosuCAPA-DHr	QYAMINEQVN 429
DromeCAPA-DHr	QYAMIGVQVN 430
	***** **

Fig 5