Identification and Expression of Capa Gene in the Fire Ant, Solenopsis invicta

Man-Yeon Choi1*, Rene Köhler2, Robert K. Vander Meer1, Susanne Neupert2, Reinhard Predel2*

1 United States Department of Agriculture-Agricultural Research Service (USDA-ARS), Center of Medical, Agricultural and Veterinary Entomology (CMAVE), Gainesville, Florida, United States of America, 2 Zoological Institute, Biocenter University of Cologne, Cologne, Germany

Abstract

Recent genome analyses suggested the absence of a number of neuropeptide genes in ants. One of the apparently missing genes was the capa gene. Capa gene expression in insects is typically associated with the neuroendocrine system of abdominal ganglia; mature CAPA peptides are known to regulate diuresis and visceral muscle contraction. The apparent absence of the capa gene raised questions about possible compensation of these functions. In this study, we re-examined this controversial issue and searched for a potentially unrecognized capa gene in the fire ant, Solenopsis invicta. We employed a combination of data mining and a traditional PCR-based strategy using degenerate primers designed from conserved amino acid sequences of insect capa genes. Our findings demonstrate that ants possess and express a capa gene. As shown by MALDI-TOF mass spectrometry, processed products of the S. invicta capa gene include three CAPA periviscerokininins and low amounts of a pyrokinin which does not have the C-terminal WFGPRLa motif typical of CAPA pyrokinins in other insects. The capa gene was found with two alternative transcripts in the CNS. Within the ventral nerve cord, two capa neurons were immunostained in abdominal neuromeres 2–5, respectively, and projected into ventrally located abdominal perisym pathetic organs (PSOs), which are the major hormone release sites of abdominal ganglia. The ventral location of these PSOS is a characteristic feature and was also found in another ant, Atta sexdens.

Introduction

Although higher insect taxa (“insect order” diverged more than 200 mya, general features of the hexapod neuroendocrine system, including cell location, hormone release sites, and sequences of the peptide hormones are strongly conserved. This conservation reflects the functional significance of these peptide messenger molecules. Therefore, it was unexpected that recent genome analyses failed to identify several neuropeptide genes in a number of insects. For the key substances of the segmentally arranged perisym pathetic organs (PSOs), which store products of the extended fnaf gene in the thorax and products of the capa gene in the abdomen, this situation was first described from the parasitic wasp Nasonia vitripennis (Hymenoptera; [1]). Here, the absence of otherwise highly conserved peptide hormones could be attributed to the miniaturization of the nervous system or parasitic lifestyle but later it was described that ant genomes do not possess fnaf and capa genes as well [2].

We addressed this question with a simple approach and used a CAPA antiserum raised against a cockroach CAPA peptide to screen the ventral nerve cord (VNC) of the fire ant, Solenopsis invicta, for CAPA immunoreactive (ir) cells. Indeed, we observed the typical CAPA immunoreactivity in neurons of abdominal ganglia and identical cells were immunostained in other ant species as well. These findings were the reason to search for the potentially overlooked capa gene from S. invicta. To achieve this aim, we employed a PCR-based strategy using degenerate primers designed from conserved amino acid sequences of capa genes, and searched for similar genes from the fire ant genome [3]. In the present study we demonstrate the identification, structure and characterization of the capa gene, transcription profiles, translated mature peptides, and location of capa neurons in the abdominal VNC and attached release sites. The distribution of capa neurons in the VNC of S. invicta is compared with that in another ant, Atta sexdens, and the honey bee, Apis mellifera.

Material and Methods

1 Insects

Fire ants, S. invicta, were taken from monogyne (single egg laying queen) colonies collected in the Gainesville area in Florida, USA. Colonies collected by excavation were removed from the soil by floating out the workers and brood as previously described [4]. No specific permits were required for the described field collections and the collections did not affect endangered or protected species. All colonies were fed crickets and 10% sugar solution absorbed onto wads of tissue and maintained under standard laboratory conditions. Leaf-cutter ant, A. sexdens, was provided by Wolfgang Rossler (Würzburg, Germany), and A. mellifera was provided by Elke Woker (Jena, Germany). All animal procedures were

* E-mail: mychoi@ars.usda.gov (MYC); rpredel@uni-koeln.de (RP)

Current address: United States Department of Agriculture-Agricultural Research Service, Horticultural Crops Research Unit, Corvallis, Oregon, United States of America.

Citation: Choi M-Y, Köhler R, Vander Meer RK, Neupert S, Predel R (2014) Identification and Expression of Capa Gene in the Fire Ant, Solenopsis invicta. PLoS ONE 9(4): e94274. doi:10.1371/journal.pone.0094274

Editor: Wolfgang Blenau, Goethe University Frankfurt, Germany

Received November 17, 2013; Accepted March 14, 2014; Published April 9, 2014.

This is an open-access article, free of all copyright, and may be freely reproduced, distributed, transmitted, modified, built upon, or otherwise used by anyone for any lawful purpose. The work is made available under the Creative Commons CC0 public domain dedication.

Funding: The authors acknowledge the financial assistance of the Deutsche Forschungsgemeinschaft (Predel 595/6-1, 2; 766/9-1) and Luxemburg Foundation (to RK). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.
conducted in compliance with protocols approved by local government authorities and were in accordance with National Institutes of Health guidelines.

2 Molecular cloning and characterization
Poly [A]⁺ RNA was isolated from winged females without head by Micro Fast mRNA purification kit (Invitrogen, CA, USA) and used to synthesize cDNA with the GeneRacer cDNA synthesis kit (Invitrogen). First we designed degenerate primers based on conserved amino acid sequences (MWFGPRLG) for sense primers, 5'-ATGTGGCTTGGGNCCNGNYTTGGG-3' and 5'-TGGTTYGGNCNGMNMGKYNTNGG-3', and antisense primers, 5'-CCNARNCKNNGNCRAACCACT-3' and 5'-CCNARNCKNNGGCRAACAGC-3'. Those primers were used for PCR amplification with cDNA synthesized from winged females without head using 5' and 3'-Rapid Amplification of cDNA End (RACE) method (Invitrogen) using manufacturer's procedure. Secondly, BLAST was employed to search the S. invicta genome [3] with known insect capa genes from GenBank. Specific forward and reverse primers, 5'-TAC-CAAGA CTAGGTCGCGAGAGTTGA-3' and 5'-TGA ATCCTTGAGGAGAAAACG-3' were designed based on the amino acid sequence YTTPRLGRESE found in the S. invicta genome. Those primers were used for 3'- and 5'-RACE, respectively, and PCR amplification using the manufacturer's procedure (Invitrogen). RACE PCR products were checked using 1.5% agarose gel electrophoresis and visualized using GelRed (Biotium, CA, USA) under a UV light. The PCR products were then purified, cloned into a subcloning vector (TOPO-TA, Invitrogen), sequenced by Sanger DNA sequencing (Interdisciplinary Center for Biotechnology Research, ICBR, University of Florida, Gainesville, FL, USA), and analyzed by Genetyx DNA software (ver. 10, Genetyx Co., Tokyo, Japan). Resulting sequences were used to design another primer set: 5'-CAGGACAACCGG-3' and 5'-TGTCCGAGATGATCGTCACCAG-3' to synthesize the full sequence of the fire ant capa gene. PCR was performed for 35 cycles at 95°C for 30 s, 63°C for 30 s, and 72°C for 1 min, then 72°C for 5 min using iTaq DNA polymerase (Bio-Rad, CA, USA). PCR products after gel purification were subcloned into the subcloning vector described above and analyzed by DNA sequencing. We found a 5-7-Kb long DNA sequencing contig containing exons and introns of the capa gene (see Figure S1) in the S. invicta genome data using the obtained capa mRNA sequence and analyzed by Genetyx DNA software.

3 Reverse transcriptase (RT)-PCR for capa expression
Total RNA was isolated from the following fire ant samples: fertilized eggs, female larva, female pupa, and workers without head; in addition head, brain, subesophageal ganglion (SEG), and VNC of workers, using the PureLinkTM RNA Kit (Invitrogen). RNA was quantified by a NanoDrop 2000 (Thermo Scientific, PA, USA) and used to synthesize cDNA using SuperScript RT III according to the manufacturer’s protocol (Invitrogen). The first-strand cDNA synthesized from the different tissues was used for PCR amplification with a primer set: 5'-CACCTGTTGCACAGAATGCGAGCAACCGG-3' and 5'-TGTTGGTGCCCTATGGAATAGGAGAAAACG-3' to amplify a full sequence of the fire ant capa gene. PCR were performed for 35 cycles at 95°C for 30 s, 63°C for 30 s, and 72°C for 50 s, then 72°C for 5 min using iTaq DNA polymerase (Bio-Rad). Prepared PCR products were checked using 1.5% agarose gel electrophoresis, purified and the sequences confirmed by DNA sequencing as described above.

4 Mass spectrometry

Dissection and sample preparation for mass spectrometry. Immobilized large workers and/or reproducives of S. invicta were fixed with insect pins and completely submerged in insect saline (in mM: NaCl 126, KCl 5.4, NaH₂PO₄ 0.17 and KH₂PO₄ 0.22; pH 7.4). Subsequently, the thorax and abdomen (including the petioles) were dorsally opened with an ultrasonic scissor and, after removal of the gut, the VNC was dissected and transferred into a separate dish containing saline. The tiny abdominal PSOs were visualized and manually dissected with a high resolution stereomicroscope (Lumar V12; Carl Zeiss, Göttingen, Germany), and transferred with a glass capillary in a drop of purified water on the sample plate for MALDI-TOF mass spectrometry. The water was removed using the transfer glass capillary before the dried samples were covered with a mixture of 0.2 µl 2-cyano-4-hydroxycinnamic acid (CHCA, Sigma-Aldrich, Steinheim, Germany) dissolved in 50% methanol. After drying at room temperature, each spot was finally rinsed with water for a few seconds.

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. Mass spectrometric analysis was performed on an ABI 4800 proteomics analyzer (AB Sciex, Darmstadt, Germany). The acquisitions were taken in manual mode. Instrument calibration was performed using the peptide standard kit 1 (Bruker Daltonik GmbH, Bremen, Germany). Initially, the instruments were operated in reflectron mode, in order to determine the parent masses. For the tandem MS experiments, we used PSD as well as CID mode. The fragmentation data obtained in these experiments were analyzed with Data Explorer 4.3 (AB Sciex) and used to confirm the sequences of the fire ant neuropeptides [6].

5 Immunocytochemistry and documentation
VNCs of adults were fixed for 3 h at 4°C with 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.2. Subsequently, samples were washed 3 times for 15 min in PBS 1% Triton X-100 at room temperature. The preparations were then incubated for 2 days at room temperature in anti-CAPA-periviscerokinin-2 (PVK-2) serum (1:4000), which was raised against Periplaneta americana CAPA-PVK-2 (GSSSGLISMPRV-NH₂) [7], diluted with PBS 1% Triton X-100 containing 0.25% bovine serum albumin and 10% normal goat serum. Following washing in PBS 1% Triton X-100 for 24 h, the preparations were incubated with the secondary antibody conjugated to Cy3 (1:3000; Jackson ImmunoResearch, Suffolk, UK) in PBS for 2 days at room temperature. Finally, the preparations were washed again for 24 h in PBS 1% Triton X-100 and mounted in Mowiol (Calbiochem, Darmstadt, Germany). Immunostainings were examined with a confocal laser scanning microscope (ZEISS LSM 510 Meta system, Jena, Germany), equipped with a Plan-Neofluar 40x/1.3 oil objective and a HeliumNeon1 laser (wavelength 543 nm). Serial optical sections were assembled into combined images. Images were exported and processed (contrast, brightness, image detail, file format) with Adobe Photoshop 7.0 software. For the scale drawings, printouts of the LSM scans were traced onto tracing paper using a technical pen. For a correct assignment of the 3D position of immunostained cells within the ganglion, preparations were examined with a fluorescence stereomicroscope (Lumar V12) during drawing.
Figure 1. *S. invicta* capa mRNA transcripts, translated amino acids, and schematic overview of the two splice variants. A) Long transcript. B) Short transcript. *S. invicta* PVK-1, –2, –3, and PK neuropeptides (underlined) are predicted using putative endoproteolytic cleavage sites (bold italic); Gly as potential amidation signal is italicized. The sequence between the signal peptide and PVK-1 is indicated as N-terminal precursor peptide (NPP; grey shaded).

The sequence of the *capa* gene including exons and introns is given in the File S1. GenBank accession numbers of the mRNA transcripts are KJ020267 (long form) and KJ020268 (short form).

doi:10.1371/journal.pone.0094274.g001
Results

1 Capa mRNA and precursor sequences

Two splice forms of *S. invicta* capa mRNA were identified (Fig. 1). The long transcript (591 nucleotides) and the short transcript (549 nucleotides) encode 196 amino acids and 182 amino acids, respectively. Both proteins contain six potential endoproteolytic cleavage sites [8,9], R\(^{44}\)-R\(^{45}\), R\(^{57}\), R\(^{67}\), R\(^{79}\), R\(^{172}\), R\(^{186}\) (Fig. 1A; numbering refers to the long transcript). Four of the predicted mature peptides have a C-terminal amide group provided by glycine. Three of these peptides, SAGLVAYPRI-NH\(^2\), KSDLFPRL-NH\(^2\), and TFGIIQKPRV-NH\(^2\) are considered putative CAPA-PVKs, and NSQGQGGYTPRL-NH\(^2\) shows sequence similarity with PK peptides. The C-terminal motif WFGPRL-NH\(^2\), which is typical of CAPA-PKS in most insects studied so far [10], is not present in the CAPA precursor sequence.

2 Capa gene structure

*S. invicta* capa pre-mRNA forms alternative RNA splice variants to generate two transcripts that are a long form incorporating five exons and a short form incorporating four exons from 40 kilonucleotides (nt) entire genomic DNA (see Figure S1). The splice variants show a 42-nt difference but the predicted mature CAPA-PVK/PK peptides are not different (Fig. 1). The three PVKs and the PK of the fire ant *capa* gene are encoded on exon 3 and 4. Exon 2 (42-nt) is skipped by alternative 5\(^{\text{\text{′}}/\text{"}}\) splicing in the short form where an intron gap (2-Knt) exists between exons 1 and 3 (Figs. 1C and S1).

3 Capa gene transcription

*Capa* mRNA was expressed in all developmental stages which have been studied (Fig. 2) but the transcription level was weak in the embryo. A particularly strong expression of *capa* mRNA was found in late pupae and adult insects. Both mRNA transcripts were detected in the CNS from larvae to adults while only the long form was observed in embryonic (egg) tissue. The ratio of both transscripts changed during the postembryonic development. In larval and early pupal stages as well as in headless adult ants, the expression of the two RNA forms was rather similar. In late pupae and adult head samples, the long transcript was found to be more abundant (Fig. 2A). A comparison of *capa* gene transcription in different parts of the CNS of adult females indicated that the long transcript is more abundant in brain and SEG whereas the short form is slightly more abundant in abdominal ganglia (Fig. 2B).

4 Identification of capa gene products by mass spectrometry

Direct tissue profiling of single abdominal PSOs of adult *S. invicta* yielded highly reproducible mass spectra in the mass range...
of m/z 750–2700 (Fig. 3A). The most prominent ion signals indicated the presence of the three predicted CAPA-PVKs and N-terminal precursor peptide (NPP) of the long transcript, preceding the CAPA-PVK-1 sequence (SVGQNYEPTREGQKLKIND-OH; see Table 1). The predicted NPP of the short transcript sequence (pQ/QKLKIND-OH) was not observed. Sequences of PVKs and NPP of the long transcript were confirmed by fragment analyses (see Fig. 3B for CAPA-PVK-2). In contrast to the PVKs and NPP, the predicted PK (NSQGQGGYTPRL-NH2) was identified in mass spectra with very low ion signal intensity. However, a substance mass similar with a hypothetical C-terminal precursor peptide (CPP), consisting of PK and the remaining C-terminal amino acids from the CAPA precursor (NSQGQGGYTPRL-GRESEHDAAANFP-OH), was detected. Ion signal intensity of this peptide was not sufficient for MS/MS experiments.

Due to C-terminal sequence similarity of products from the capa gene and products from the pk/pban gene, PVK and PK/PBAN antisera both recognize products of the two genes. In our experiments, no product of the pk/pban gene could be detected in mass spectra of abdominal PSOs and abdominal VNC. PK/pban gene transcription was also not observed in the abdominal ganglia (see Fig. 2). Thus, PK/PBAN immunoreactivity in neurons of abdominal ganglia [5] can be attributed to the CAPA peptides. Conversely, mass spectra obtained from corpora cardiaca of S. invicta did not indicate the presence of CAPA-PVKs or PK from the capa gene. Instead, all predicted PKs [5] and the pyroglutamate form of the CPP from the pk/pban gene (pQLRNVLRK-L-OH) were observed in mass spectra from corpora cardiaca (Fig. 4; Table 1).

### Table 1. List of peptides from capa and pk/pban genes of S. invicta, identified by MALDI-TOF/TOF mass spectrometry in this study.

| peptide name | sequence | m/z | aPSO | CC |
|--------------|----------|-----|------|----|
| CAPA-peptides |          |     |      |    |
| long NPP     | SVGQNYEPTREGQKLKIND-OH | 2176.10 | x | - |
| PVK-1        | SAGLVAYPRI-NH2         | 1045.62 | x | - |
| PVK-2        | KSDLFPRL-NH2           | 974.58  | x | - |
| PVK-3        | TFGIQQKRPRV-NH2        | 1157.72 | x | - |
| PK           | NSQGQGGYTPRL-NH2       | 1276.64 | x | - |
| CPP          | NSQGQGGYTPRLEGRESDAANFP-OH | 2588.19 | x | x |
| PK/PBAN      |          |     |      |    |
| PK-1         | TSQDIASGMWFPRPRL-NH2  | 1664.82 | - | x |
| PK-2         | pQPQFPPRPL-NH2         | 968.53  | - | x |
| PK-3         | GSGEDLSYGAYEVDHHPLVPRL-NH2 | 2894.31 | - | x |
| PK-4         | RLWPWLPRL-NH2          | 1233.76 | - | x |
| CPP          | pQLRNLRLK-L-OH         | 1122.71 | - | x |

CPP, C-terminal precursor peptide; NPP, N-terminal precursor peptide; PBAN, pheromone biosynthesis activating neuropeptide; PVK, periviscerokinin; PK, pyrokinin; aPSO, abdominal PSO; CC, corpora cardiaca.

1. mass match only.
doi:10.1371/journal.pone.0094274.t001

5 Localization of CAPA immunoreactivity in the VNC of S. invicta and related Hymenoptera

5.1 S. invicta. The VNC of adult S. invicta consists of three thoracic ganglia with the ganglion in the metathorax composed of thoracic neuromere 3 and abdominal neuromeres 1+2, three unfused abdominal ganglia, and the terminal ganglion. The first two unfused abdominal ganglia are located in the first and second node of the petiole, respectively. Immunostainings with the P. americana PVK-2 antiserum yielded CAPA-ir neurons in abdominal neuromeres 2–5; somata of these cells were always located ventrally. Abdominal neuromere 1, which is generally fused with the metathoracic ganglion in pterygote insects [11], did not contain CAPA-ir cells. Abdominal neuromere 2 showed a bilateral pair of CAPA-ir neurons which cell bodies were located posteromedially. The axons of the CAPA-ir cells first projected together dorsomedially into the anterodorsal midline of the neuromere before passing to the ventral side and entering an anterovertrally located bulblike PSO. A similar projection of CAPA-ir neurons was found in the three unfused abdominal ganglia.
ganglia although the position of cell bodies varied from a typical posteromedian location to a more anterolateral location in a few cases. In contrast to the CAPA-ir fibers in abdominal neuromere 2, the axons of the CAPA-ir neurons in unfused abdominal ganglia usually crossed the neuromere separately before joining in the midline of the dorsal cortex (Fig. 5A). In 2 out of 15 analyzed unfused abdominal ganglia, the bulb- or tonguelike PSOs were not located at the ventral side but at the opposite side at the anterodorsal margin of the ganglion. The terminal ganglion (with abdominal neuromeres 6–11) did not show CAPA-ir neurons. An overview of the CAPA-ir neurons in the VNC of adult *S. invicta* is given in Fig. 5.

### 5.2 A. sexdens.

The VNC of adult *A. sexdens* consists of three thoracic ganglia with the ganglion in the metathorax composed of thoracic neuromere 3 and abdominal neuromeres 1+2, only two unfused abdominal ganglia, and the terminal ganglion. The first unfused abdominal ganglion is located in the first node of the petiole, the second abdominal ganglion is located in the gaster immediately posterior of the petiole. Immunostainings of the VNC of *A. sexdens* with the *P. americana* PVK-2 antiserum yielded six abdominal neuromeres (2–7) with CAPA-ir neurons. Somata of these cells were always located ventrally. In abdominal neuromere 2, immunostainings revealed a cell location and projection very similar to those described above for *S. invicta*. In the two unfused abdominal ganglia (abdominal neuromeres 3, 4), the initial projection of the posteromedially located CAPA-ir somata is also nearly identical with the projection of CAPA-ir cells in *S. invicta*, but the axons of the two CAPA-ir cells joined each other already before projecting dorsally. Different from the situation observed in *S. invicta*, the respective abdominal PSOs were not located in an anterior position but posterovertral. Therefore, after reaching the midline of the anterovertral cortex of the ganglion, the CAPA-ir fibers curved posteriorly and crossed the ganglia ventral to the CAPA-ir somata before entering the respective PSOs. The terminal ganglion (with abdominal neuromeres 5–11) showed three neuromeres (abdominal neuromeres 5–7) with CAPA-ir neurons. As in the unfused abdominal ganglia, the respective PSOs were clearly located posterovertrally of the CAPA-ir somata; cell location and projection were similar to those described for the unfused ganglia. An overview of the CAPA-ir neurons in the VNC of *A. sexdens* is given in Fig. 6.

### 5.3 A. mellifera.

The VNC of female *A. mellifera* consists of thoracic ganglion 1, a ganglionic mass consisting of thoracic neuromere 3 and abdominal neuromeres 1+2, three unfused abdominal ganglia (with abdominal neuromeres 3–5), and two fused abdominal ganglia (abdominal neuromeres 6, 7) prior to the terminal ganglion (abdominal neuromeres 8–11). Immunostainings with the *P. americana* PVK-2 antiserum yielded six abdominal neuromeres (2–7) with two ventral CAPA-ir cell bodies each.
Axons of these posteromedially located somata projected anterodorsally. In the dorsal midline of abdominal neuromeres 3–6, the axons then projected together or slightly separated anteriorly and innervated PSOs which were more or less protruding from the dorsal anteromedian ganglionic sheath (Fig. 7A). In neuromeres 2 and 6, the neurites of the capa cells enter PSOs which are usually located opposite to the somata at the dorsal ganglionic sheath. Particularly the PSO of neuromere 7 showed variability regarding shape and position along the longitudinal axis of the ganglion; ranging from the development of PSOs typical of the more anterior abdominal ganglia (Fig. 7B) to the development of well separated PSO (Fig. 7C).

Discussion

CAPA-peptides are typically synthesized from the neuroendocrine system of the abdominal VNC in insects [10]. Expressed in at least two ventral neurosecretory cells of abdominal ganglia (Va-neurons in Drosophila melanogaster [12,13], VL-cells in P. americana [14], NS-M4 neurons in Manduca sexta [15,16]), these peptides are transported into neurohemal release sites that are usually developed as distinct abdominal PSOs [17]. The mature neuropeptide species (CAPA-PVKs and CAPA-PK) processed from the CAPA precursor were structurally first identified in the cockroach P. americana [18,19]. Current available genome data, sequence similarities of processed peptides and the corresponding receptors suggest a common ancestor of capa and pk genes. Possibly within the Pancrustacea lineage, a gene duplication of this predecessor occurred which, in turn, might have a common ancestor with neuregulin U of vertebrates [20]. In insects, the pk gene is known by different names (e. g. pban of Lepidoptera, hugin of Drosophila) but is always expressed in neurosecretory neurons of the SEG [5,21–25] which is different from the major expression site of the capa gene. CAPA-PVKs and CAPA-PK (C-terminal WFGPRLamide) both have specific receptors [26,27].
Figure 8. Hypothetical development of the architecture of abdominal PSOs in some hymenopteran insects. Arrows do not indicate phylogenetic lineages. A) Plesiomorphic situation as e. g. in the cockroach *P. americana* [17] with CAPA peptide accumulating PSOs at the junction of median nerve/transverse nerves. B) Intermediate development as e. g. in *Vespula germanica* (Vespidae, Aculeata); transverse nerves are no longer linked with the median nerve. CAPA peptide accumulating PSOs are club-shaped modifications of the anterior median nerve (Predel et al., unpublished). C) In *A. mellifera* (Apidae, Aculeata), the remains of the median nerve are more or less incorporated in the anterodorsal ganglionic sheath; the neurohemal nature of this tissue is well visible in anti-CAPA immunostainings (see Fig. 7). D) Ants (Formicidae, Aculeata) possess abdominal PSOs that are as distinct as in *Vespula* but their location is moved from an anterodorsal position to an anteroventral or (in *A. sexdens*) even to a posteriorventral position, which is unique in insects.

doi:10.1371/journal.pone.0094274.g008

CAPA-PVKs are known to stimulate visceral muscle contraction and regulate the activity of Malpighian tubules in many insects [10,28]. However, the function of CAPA-PK is largely unknown. The *capa* gene was missed in recent genomic analyses of the ants *Acromyrmex echinatior* [2], *Camponotus floridanus* [29], *Harpegnathos saltator* [29], and *S. invicta* [3] and also in the parasitic wasps *Nasonia vitripennis*, *N. giraulti*, and *N. longicornis* [1]. This absence raised questions about possible compensation of functions of CAPA peptides. In previous studies on *S. invicta* [5,30], distinct PK/PBAN immunoreactivity was observed in abdominal ganglia and associated PSOs. However, *pk/pban*-mRNA transcription was not detectable in these tissues (see also Fig. 2), which suggested expression of sequence-related peptides from another gene. The candidate gene was the hitherto unidentified *capa* gene; subsequent immunostainings by means of a CAPA-PVK (PKamide-based) antiserum demonstrated a cellular pattern identical with that obtained with the PK/PBAN antiserum [5]. The presence of these immunostainings in abdominal ganglia was thus a convincing argument to specifically search for the *capa* gene in *S. invicta*.

Our experiments demonstrated that ants possess and express a *capa* gene and alternative transcripts in the CNS. Alternative splicing of neuropeptide gene transcripts arise from differential use of splice sites localized within the gene transcript domain and skips single or multiple exons to modify the presence or sequence of mature peptides. The long and short *capa* transcripts, which were similarly abundant in the VNC of *S. invicta*, differed by 42-nt. This alternative splicing does not affect the translation of CAPA-PVK and PK peptides, but the short form of the precursor contains a truncated NPP sequence (QKLKIND; long form SVGQNYEP-TREGQKLKIND). This peptide (pQ/QKLKIND) was not observed in mass spectrometric analyses. Examples of alternative mRNA transcript splicing were found in e. g. human genes [31], insect neuropeptide genes [32–35], and neuropeptide receptor genes [36–38]. However, the well established differential distribution of CAPA peptides in the CNS of *D. melanogaster* (Va-neurons/abdominal PSOs: PVKs, CAPA-PK, CPPB; SEG/retrocerebral complex: only CAPA-PK and CPPB [39]) and other insects (e. g. *Aedes aegypti*, [40], *Rhodius prolixus* [41], *P. papatasi* [42]) likely resulted from differential precursor processing. No evidence for multiple *capa* mRNA transcripts was found for these species.

The number of CAPA-PVK and PK peptides translated from *capa* genes of holometabolous insects is different between taxa (see Figure S2). Regarding conserved neuropeptides with substantiated receptor activation, the most significant difference between hymenopteran species and other insects is the absence of a CAPA-PK sequence in Hymnoptera with a C-terminal motif WFGPRLa. In several insect species two PKs are encoded in the *capa* gene, one with the WFGPRLa-motif and another one with a C-terminal YXPRLa-motif. Since both of these PK forms are also encoded in the *capa* gene of the cockroach [43], the presence of two forms of PKs is likely a plesiomorphic character in insects which was also observed in holometabolous insects such as *A. aegypti* [40] and *Tribolium castaneum* [44] but not in *A. mellifera* [45]. In *D. melanogaster* (and other insects; see Figure S2) the second PK with a C-terminal YXPRLa-motif has been lost and the remaining CAPA-PK belongs to the WFGPRLa-type. This PK activates, in *D. melanogaster*, a specific receptor (PK-1 receptor [26]). The *capa* gene of *S. invicta* represents the first *capa* gene identified in insects, whose sequence conserved the PK of the YXPRLa-type but lost the PK of the WFGPRLa-type. Therefore, the *S. invicta* CAPA-PK is likely not ligand of a WFGPRLa-receptor but rather activates homologs of the *D. melanogaster* PK-2 receptors [46]. A further study is needed to confirm this receptor activation. Mass spectra obtained from PSO preparations confirmed, that the *S. invicta* PK is at least partially cleaved from the C-terminal precursor. The
abundance of *S. invicta* PK in abdominal PSOs was, however, much lower than the abundance observed for the PVKs.

The location of *capa* neurons (Va-neurons) in abdominal ganglia of *S. invicta* is typical of holometabolous insects. However, the position of abdominal PSOs, which store these peptide hormones, is underrepresented. Axons of the two ventral capa expressing Va-neurons first project dorsomedially into the anterodorsal midline of the respective neurone; without showing the distinct loop around the midline that is known from many insects [10,13]. Unlike in all other insects studied so far (including *A. mellifera* in this study), the axons then pass to the ventral side of the ganglion and enter an anteroventrally located PSO. Only in a few cases and as an obvious reversion to the plesiomorphic formation, we observed anterodorsally located PSOs at abdominal ganglia. In *S. invicta*, only four ganglia (those with abdominal neuromeres 2–5) exhibited CAPA-ir neurons. These ganglia included the three unfused abdominal ganglia but not the terminal ganglion. To test the generality of the observed features, particularly of the ventral position of abdominal PSOs, we studied with *A. sexdens* a second ant species which belongs to the same subfamily (Myrmicinae). Notably we observed not only ventrally located abdominal PSOs in *A. sexdens* but these PSOs were found invariably in a posterior position. Thus, axons of Va-neurons from *A. sexdens* first project dorsomedially into the anterodorsal midline of the neurones, then pass to the ventral side of the ganglia (as in *S. invicta*), curve posteriorly and cross the ganglia along the midline and ventral to the CAPA-ir somata before entering posteroventrally located PSOs. These findings suggest a position of abdominal PSOs of *S. invicta* intermediate between the original location in insects along the anterior median nerve/transverse nerve (“primitive type” of abdominal PSOs; [17]) and the highly derived posteroventral position of abdominal PSOs in *A. sexdens* (see Fig. 8). The comparison of CAPA immunostainings from *S. invicta* and *A. sexdens* revealed another interesting point with the number of segments developing Va-neurons. Whereas in *S. invicta* (3 unfused abdominal ganglia) only four neurones retained Va-neurons with *capa* gene expression, *A. sexdens* (2 unfused abdominal ganglia) showed six abdominal neurones with capa expressing Va-neurons. Thus, Hymenoptera not only show great variability in the number of abdominal ganglia [47] but also variability in the number of *capa* expressing neurones and this development does not depend on the number of unfused ganglia. From *D. melanogaster*

it is known that Va-neurons are initially generated in all VNC segments before they develop a different identity anterior to the second abdominal neurone and undergo segment-specific programmed cell death in posterior segments [48,49]. In the two ant species studied, a similarly programmed cell death of Va-neurons could therefore affect a different number of posterior neurones.

In summary, this study describes *capa* gene expression, processing and localization of CAPA peptides in the VNC of *S. invicta* and demonstrates that non-detection of genes by bioinformatic analyses of genome data does not necessarily mean that the genes are absent.

**Supporting Information**

**Figure S1** Nucleotide sequence and translated amino acids of fire ant *capa* gene. *S. invicta* PVK-1, −2, −3, and PK neuropeptides (underlined) are predicted using putative endoproteolytic cleavage sites (bold italic). Alternative splicing nucleotide (42-nt; highlighted in gray) correspond to exon 2. Expected TATA box as promoter binding site, the first ATG as the initiation codon, and TAA as the termination codon are indicated with boxes. (DOCX)

**Figure S2** Translated amino acid sequences of selected *capa* genes from holometabolous insects. Note that the C-terminal sequence of the CAPA precursor from *A. mellifera* is different from the sequence published by Hummon et al. [45]. (DOCX)

**Acknowledgments**

We also thank David Milne (USA) for technical support, DeWayne Shoemaker (USA) for the genome searching, James Becnel and Steven Valles (USA) for reviewing the early version of the paper, Jörg Kahnt (Germany) for support during the mass spectrometric analysis, and Wolfgang Rössler (Germany) for providing specimens of *Atta sexdens*.

**Author Contributions**

Conceived and designed the experiments: MYC RP. Performed the experiments: MYC RP RK SN. Analyzed the data: RP RK MYC SN RKVM. Contributed reagents/materials/analysis tools: RKVM. Wrote the paper: RP MYC.

**References**

1. Hauser F, Neupert S, Williamson M, Predel R, Tanaka Y, et al. (2010) Genomics and peptidomics of neuropeptides and protein hormones present in the parasitic wasp *Nasonia vitripennis*. J Proteome Res 9:5296–5310. doi:10.1021/ pr100579q.

2. Nygaard S, Zhang G, Schiøtt M, Li C, Wurm Y, et al. (2011) The genome of the leaf-cutting ant *Atta sexdens* suggests key adaptations to advanced social life and fungus farming. Genome Res 21:1339–1348. doi:10.1101/ gr.121392.111.

3. Wurm Y, Wang J, Riha-Grognuz O, Corona M, Nygaard S, et al. (2011) The genome of the fire ant *Solenopsis invicta*. Proc Natl Acad Sci U S A 108:5679–5684. doi:10.1073/pnas.1005370.

4. Banks WA, Lodgren CS, Jouvenaz DP, Stringer CE, Bishop PM, et al. (1981) Techniques for collecting, rearing, and handling imported fire ants. USDA, SEA, AATS-S-21, 9 p.

5. Choi MY, Vander Meer RK, Shoemaker D, Valles SM (2011) PBAN gene architecture and expression in the ant, *Solenopsis invicta*. J Insect Physiol 57:161–165.

6. Wegener C, Neupert S, Predel R (2010) Direct MALDI-TOF mass spectrometric peptide profiling of neuroendocrine tissue of *Drosophila*. Mol Biol 61:117–127. doi:10.1007/s00018-010-0676-1-65.

7. Predel R, Rapsin J, Eckert M, Holman GM, Nachman RJ, et al. (1998) Isolation of periviscerokinin-2 from the abdominal perisynaptic organs of the American cockroach, *Periplaneta americana*. Peptides 19:801–809.

8. Southey BR, Swedler JV, Rodriguez-Zas SL (2000) Prediction of neuroepitope cleavage sites in insects. Bioinformatics 24:915–925. doi:10.1093/bioinformatics/24.11.915.

9. Veerstra JA (2000) Mono- and dibasic proteolytic cleavage sites in insect neuroendocrine peptide precursors. Arch Insect Biochem Physiol 43:49–63. doi:10.1002/(SICI)1520-6327(200002)43:2<4::AID-AEC2>3.0.CO;2-J.

10. Predel R, Wegener C (2006) Biology of the CAPA peptides in insects. Cell Mol Life Sci 63:2477–2490. doi:10.1007/s00018-006-6187-3.

11. Niven JE, Graham CM, Burrows M (2008) Diversity and evolution of the insect ventral nerve cord. Ann Rev Entomol 53:253–271. doi:10.1146/annurev-ento-52110405091322.

12. Kuan L, Cazeneve W, Costes L, Broderick KE, Graham S, et al. (2002) Two nitride peptides are encoded by the gene capability in *Drosophila melanogaster*. Am J Physiol Regul Integr Comp Physiol 282.R1297–1307. doi:10.1152/ajpregu.00304.2001.

13. Santos JS, Pollak E, Rexer KH, Mokran L, Wegener C (2006) Morphology and morphogenesis of the peptidogenic Va neurons and the median nerve system of the fruit fly, *Drosophila melanogaster*. Cell Tissue Res 326:167–199. doi:10.1007/s00441-006-0211-7.

14. Eckert M, Predel R, Gundel M (1999) Periviscerokinin-like immunoreactivity in the nervous system of the American cockroach. Cell Tissue Res 295:159–170.

15. Predel R, Herbert Z, Eckert M (2003) Neuropeptides in perisynaptic organs of *Manduca sexta* specific composition and changes during the development. Peptides 24:1457–1464.

16. Lei PK, Tublitz NJ (2004) Sequence and expression of the CAPA/CAP2b gene in the tobacco hawkmoth, *Manduca sexta*. J Exp Biol 207:3681–3691. doi:10.1242/jeb.01186.
Capa Gene Expression in the Ant

17. Raabe M (1983) The neurosecretory-neurohaemal system of insects: anatomical, structural and physiological data. Adv Insect Physiol 17:205–303.

18. Predel R, Linde D, Raps J, Vettermann S, Penzlin H (1995) Perিমিকενωνεια (Pen-PK): a novel myotropic neuropeptide from the perisynaptic organs of the American cockroach. Peptides 16:61–66.

19. Predel R, Kelher R, Nachman RJ, Holman GM, Raps J, et al. (1999) Differential distribution of pyrinokin-isomers in cerebral and abdominal neurohemal organs of the American cockroach. Insect Biochem Mol Biol 29:139–144.

20. Melcher C, Baden R, Walther S, Simakov O, Pankratz MJ (2006) Neuropeptide U and its putative Drosophila homolog hugi. PLoS Biol 4 (5):e68. doi:10.1371/journal.pbio.0040068.

21. Davis NT, Homburg U, Teal PE, Alstein M, Agricola HJ, et al. (1996) Neuronaanatomy and immunocytochemistry of the median neuroendocrine cells of the subesophageal ganglion of the tobacco hawkmoth, Manduca sexta: immunoeasitive to PBAN and other neuropeptides. Micro Res Techn 35:201–229. doi:10.1002/mrc.2181.

22. Ma PW, Garden RW, Niermann JT, M OC, Sweedler JV, et al. (2000) Characterizing the Hev-PBAN gene products in neuronal clusters with immunocytochemistry and MALDI MS. J Insect Physiol 46:221–230.

23. Melcher C, Pankratz MJ (2005) Candidate gustatory interneurons modulating feeding behavior in the Drosophila brain. PLoS Biol 3 (9):e305. doi:10.1371/journal.pbio.0040068.

24. Predel R, Eckert M, Pollak E, Molnar L, Scheibner O, et al. (2007) Peptidomics reveals tagma-specific peptide expression and differential processing. J Neurochem 101:120–129. doi:10.1111/j.1471-4159.2007.04631.x.

25. Neupert S, Huetteroth W, Schachtner J, Predel R (2009) Conservation of the neuropeptide receptors for the Drosophila brain. PLoS Biol 3 (9):e305. doi:10.1371/journal.pbio.0040068.

26. Cazzamali G, Torp M, Hauser F, Williamson M, Grimmelikhuijzen CJ (2005) The Drosophila gene CG9918 codes for a pyrokinin-1 receptor. Biochem Biophys Res Commun 335:14–19. doi:10.1016/j.bbrc.2004.03.095.

27. Predel R, Eckert M, Pollak E, Mohan L, Schiebner O, et al. (2007) Peptidomics of identified neurons demonstrates a highly differentiated expression pattern of EPFPLamides in the neuroendocrine system of an insect. J Comp Neurol 500:490–512. doi:10.1002/cne.21183.

28. Neupert S, Huetteroth W, Schachtner J, Predel R (2009) Conservation of the function counts: homologous neurons express sequence-related neuropeptides that originate from different genes. J Neurochem 111:757–765. doi:10.1111/j.1471-4159.2009.06361.x.

29. East P, Tregenza K, Duve H, Thorpe A (1996) Identification of the dipteran capa neuropeptides. Gen Comp Endocrinol 135:195–210.

30. Choi MY, Raina A, Vander Meer RK (2009) PBAN/pyrokinin peptides in the central nervous system of the fire ant, Solenopsis invicta. J Neurochem 111:757–765. doi:10.1111/j.1471-4159.2009.06361.x.

31. Johnson JM, Castle J, Garrett-Engele P, Kazi Z, Leech PM, et al. (2003) Genome-wide survey of human alternative pre-mRNA splicing with exon junction microarrays. Science 302:2114–2114. doi:10.1126/science.1092428.

32. Taylor PA, Srd, Bhatt TR, Horodysky FM (1996) Molecular characterization and expression analysis of Manduca sexta allatotropin. Eur J Biochem 239:380–396.

33. East P, Tregenza K, Duve H, Thorpe A (1996) Identification of the dipteran Leu-callatostatin peptide family: characterisation of the prohormone gene from Calliphora vomitoria and Lucilia cuprina. Regul Peptides 67:1–9.

34. Lee KY, Chamberlin ME, Horodysky FM (2002) Biological activity of Manduca sexta allatotropin-like peptides, predicted products of tissue-specific and developmentally regulated alternatively spliced mRNAs. Peptides 23:1933–1941.

35. Horodysky FM, Bhatt SR, Lee KY (2003) Alternative splicing of transcripts expressed by the Manduca sexta allatotropin (Mas-ACT) gene is regulated in a tissue-specific manner. Peptides 22:263–269.

36. Iversen A, Cazzamali G, Williamson M, Hauser F, Grimmelikhuijzen CJ (2002) Molecular identification of the first insect ecysis triggering hormone receptors. Biochem Biophys Res Commun 299:924–931.

37. Kim YJ, Nachman RJ, Aimanova K, Gill S, Adams ME (2008) The pheromone biosynthesis activating neuropeptide (PBAN) receptor of Heliosis virescens: identification, functional expression, and structure-activity relationships of ligand analogs. Peptides 29:268–273. doi:10.1016/j.peptides.2007.12.001.

38. Lee JM, Hull J, Kawai T, Goto C, Kuribara M, et al. (2012) Re-Evaluation of the PBAN Receptor Molecule: Characterization of PBANR Variants Expresssed in the Phoroneum Glands of Moths. Front Endocrinol 3:6. doi:10.3389/fer.2012.00006.

39. Wegener C, Rein T, Jansch L, Predel R (2006) Direct mass spectrometric peptide profiling and fragmentation of larval peptide hormone release sites in Drosophila melanogaster reveals tagma-specific peptide expression and differential processing. J Neurochem 96:1362–1374. doi:10.1111/j.1471-4159.2005.06364.x.

40. Predel R, Neupert S, Garnczynski SF, Grim JW, Brown MR, et al. (2010) Neuroptidomics of the mosquito Aedes aegypti. J Proteome Res 9:2006–2015. doi:10.1021/pr901187p.

41. Neupert S, Russell WK, Russell DH, Predel R (2010) Two capa-genes are expressed in the neuroendocrine system of Blattella germanica. Peptides 31:408–411. doi:10.1016/j.peptides.2009.09.021.

42. Predel R, Neupert S, Russell WK, Hauser F, Russell DH, et al. (2013) CAPA gene products in the haematophagous sandfly Phlebotomus papatasi (Scopoli) vector for leishmaniasis disease. Peptides 41:2–7. doi:10.1016/j.peptides.2012.12.009.

43. Neupert S, Drost C, Sturm S, Predel R (2014) Identification of two capa cDNA transcripts and detailed peptidomic characterisation of their peptide products in Drosophila melanogaster. Peptides 59:125–136. doi:10.1016/j.peptides.2014.09.021.

44. Li B, Predel R, Neupert S, Hauser F, Tanaka Y, et al. (2008) Genomics, transcriptomics, and peptidomics of neuropeptides and protein hormones in the red flour beetle Tribolium castaneum. Genome Res 18:1132–122. doi:10.1101/gr.6741008.

45. Hummon AB, Richmond TA, Verleyen P, Baggerman G, Huybrechts J, et al. (2006) From the genome to the proteome: uncovering peptides in the D. mel brain. Science 314:647–649. doi:314/5799/647 [pii] 10.1126/science.1124128.

46. Rosenkilde C, Cazzamali G, Williamson M, Hauser F, Soundergaard L, et al. (2000) Molecular cloning, functional expression, and gene silencing of two Drosophila receptors for the Drosophila neuropeptide pyrokinin-2. Biochem Biophys Res Commun 289:485–494.

47. Wille-Trejos A (1961) Evolutionary trends in the ventral nerve cord of the stingless bees (Meliponinae). Rev Biol Trop 9:117–129.

48. Suska A, Miguel-Aliaia I, Thor S. (2011) Segment-specific generation of Drosophila Capability neuropeptide neurons by multi-faceted Hox cues. Dev Biol 353:72–80.

49. Gabilondo H, Losada-Pérez M, del Saz D, Molina I, León Y, et al. (2011) A targeted genetic screen identifies crucial players in the specification of the Drosophila abdominal Capaergic neurons. Mech Dev 128:201–221.