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Alternatively spliced orcokinin isoforms and their functions in *Tribolium castaneum*

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Abstract

Orcokinin and orcomyotropin were originally described as neuropeptides in crustaceans but have now been uncovered in many species of insects in which they are called orcokinin-A (OK-A) and orcokinin-B (OK-B), respectively. The two groups of mature peptides are products of alternatively spliced transcripts of the single copy gene *orcokinin* in insects. We investigated the expression patterns and the functions of OK-A and OK-B in the red flour beetle *Tribolium castaneum*. *In situ* hybridization and immunohistochemistry using isoform-specific probes and antibodies for each OK-A and OK-B suggests that both peptides are co-expressed in 5 to 7 pairs of brain cells and in the midgut enteroendocrine cells, which contrasts to expression patterns in other insects in which the two peptides are expressed in different cells. We developed a novel behavioral assay to assess the phenotypes of orcokinin RNA interference (RNAi) in *T. castaneum*. RNAi of *ok-a* and *ok-b* alone or in combination resulted in higher frequencies and longer durations of death feigning in response to mechanical stimulation in the adult assay. In the larval behavioral assays, we observed longer recovery times from knockout induced by water submergence in the insects treated with RNAi for *ok-a* and *ok-b* alone or in combination. We conclude that both OK-A and OK-B have “awakening” activities and are potentially involved in the control of circadian rhythms.

Graphical Abstract

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Awakening Activity

Keywords

neuropeptide; behavior; tonic immobility; alternative splicing

1. Introduction

Neuropeptides are a diverse family of signaling molecules that play important roles in neurotransmission and neuromodulation in animals. Homology-based searches of neuropeptides across diverse taxa have dramatically expanded the list of putative neuropeptides and revealed their evolutionary relationships via comparative studies. Most importantly, the tools of the post-genomics era have provided opportunities to explore the functions of neuropeptides in model organisms.

Orcokinin belongs to a family of myotropic neuropeptides that was originally identified in the crayfish *Orconectes limosus* as a peptide with myostimulatory activity (Stangier et al., 1992). Subsequently, these peptides were described in an expanded number of crustacean species including the following: *Carcinus maenas* (Bungart et al., 1995), *Cancer borealis* (Huybrechts et al., 2003), *Procambarus clarkii* (Yasuda-Kamatani and Yasuda, 2000), *Cherax destructor* (Skiebe et al., 2002), *Homarus americanus*, *Panulirus interruptus* (Li et al., 2002) and *Daphnia pulex* (Christie et al., 2011). Orcokinins were also described in the following insects: *Blattella germanica* (Pascual et al., 2004), *Schistocerca gregaria* (Hofer et al., 2005), *Drosophila melanogaster* (Liu et al., 2006), *Leucophaea maderae* (Hofer and Homberg, 2006a), *Locusta migratoria* (Clynen and Schoofs, 2009), *Rhodnius prolixus* (Ons et al., 2009; Ons et al., 2011), and *Bombyx mori* (Yamanaka et al., 2011). However, in insects, orcokinin does not exhibit myotropic activity (Pascual et al., 2004) but plays a role

in the control of circadian locomotor activity in the Madeira cockroach *L. maderae* (Hofer and Homberg, 2006a). In *B. mori*, orcokinin functions as an endogenous factor for ecdysteroidogenesis (Yamanaka et al., 2011).

An interesting observation is a conserved alternative splicing of the *orcokinin* gene that produces a group of related mature peptides termed OK-B that are distinct from OK-A, which denotes the typical orcokinin (Sterkel et al., 2012). In the current studies, we investigate OK-A and OK-B in the red flour beetle *Tribolium castaneum* using an RNA interference (RNAi) model system. The expression patterns were examined by reverse transcriptase (RT)-PCR, immunohistochemistry and *in situ* hybridization. The phenotypes of the exon-specific RNAi insects in the novel behavioral assays of the current study suggest that both OK-A and OK-B have “awakening” activities in *T. castaneum*.

2. Materials and Methods

2.1. Insects

The *T. castaneum* colony was maintained in a 30 °C growth chamber on a 16:8 light cycle (L:D) and fed a diet consisting of wheat flour and brewer's yeast (10:1). All experimental animals used in this study were the Georgia-1 (GA1) strain of *T. castaneum* (Haliscak and Beeman, 1983).

2.2. Identification of *Tribolium* orcokinin (TcOKA) and its alternatively spliced form (TcOKB)

A BLAST search for OKB in the *Tribolium* genome database identified the current annotation (Tcas3.0) TC005944 (Kim et al., 2010). A manual OK-A prediction was performed in the region near TC005944 and was assisted by the FGENESH program (Solovyev et al., 2006) from the Softberry website (<http://www.softberry.com>). To confirm the predicted sequences, we performed reverse transcriptase-PCR (RT-PCR). The total RNA was isolated from the entire bodies of six *T. castaneum* individuals in last larval instar using TRI reagent (Ambion). The total RNA was treated with DNase I (Ambion) to eliminate the genomic DNA and further purified by a phenol-chloroform extraction. The first-strand cDNA was synthesized with a SuperScriptTM II First-Strand Synthesis System for RT-PCR using random hexamers in a total volume 20 µL according to the manufacturer's instructions (Invitrogen Life Technologies).

The first-strand cDNA was used as a template to amplify the predicted orcokinin sequences utilizing a high fidelity polymerase PrimeSTARTM HS (Takara). The primers for orcokinin (*Tcok-a*) and its alternatively spliced form (*Tcok-b*) and all other primers are listed in Table 1. The 50 µL PCR reaction included ~50 ng cDNA, 10 µL 5x PrimeSTAR buffer with Mg²⁺, 0.32 mM of each dNTP, and 0.2 µM of each primer. The PCR was performed as follows: 35 cycles of 98 °C for 10 sec, 58 °C for 10 sec, and 72 °C for 90 sec; and a final extension of 6 min at 72 °C. The PCR product was purified using a Zymo PCR clean up kit (Zymo research) and sequenced in both directions. The nucleotide sequences and deduced amino acid sequences were analyzed using DNAMAN7 (LynnonBioSoft).

A search of orckinin sequences were conducted using BlastP against the non-redundant protein sequences (nr) database of the NCBI website (<http://www.ncbi.nlm.nih.gov/>). Sequence alignments were performed with ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). The sequence logos for the orckinin C-terminal motifs of each species were generated by Weblogo (Crooks et al., 2004).

2.3. Quantitative reverse transcriptase PCR (Q-RT-PCR)

Total RNA of the insects at different developmental stages was prepared as described previously (Begum et al., 2009). To analyze the tissue-specific expression, we collect total RNA from each of the following dissected tissues: central nervous system (CNS, including the brain and ganglia), midgut, hindgut, and carcass, excluding the aforementioned tissues. Pools of ten last-instar larvae were used to prepare the midgut, hindgut, and carcass, and twenty individuals were pooled to collect the CNS tissue. The Total RNA was treated with DNaseI (Ambion) and followed by the Phenol-Chloroform extraction. About 200ng total RNA of each tissue was used to synthesize the first-strand cDNA for qPCR by using a ImProm-II Reverse Transcription System (Promega). Quantification of the total RNA was made by a spectrophotometric method in a NanoDrop 1000 (Thermo Scientific). The primers used in the qPCR are listed in Table 1. The cDNA for the late larval stage was diluted to 1/5, 1/20, 1/80, 1/320, and 1/1280 and employed as a standard to obtain the primer efficiencies for each amplicon. The primer efficiencies were 95.2%, 93.4 % and 92.2% for RPS3, *Tcok-a* and *Tcok-b*, respectively. Q-RT-PCR was performed using the iTaq™ Universal SYBR Green Supermix (Biorad) on a CFX Connect real-time detection system (Biorad). The expression levels are shown as relative mRNA levels normalized to the reference gene *ribosomal protein S3* (*rpS3*, GenBank accession number is CB335975) (Lord et al., 2010; Sang et al., 2015) using the $\Delta\Delta CT$ method (Jiang et al., 2014). A melting curve analysis was conducted to ensure the specificity of the qPCR. We run an agarose gel for the amplicon to further confirm the specificity. The relative expressions of the target transcripts in the early eggs (EE) served as the calibrator for the developmental expression profiling, and target transcript expression in CNS was used as the calibrator for the tissue-specific expression profiling. Three biological replications were performed for each stage- and tissue-specific qPCR. The samples for studying expression levels were collected in between 1 to 6:00 PM.

2.4. In situ hybridization

Two pairs of probes each for *Tcok-a* and *Tcok-b* were designed based on the variant-specific exons. Single-stranded DNA was generated using asymmetric PCR with the primers listed in Table 1 and a DIG Probe Synthesis Kit (Roche). Sense (for the negative control) and antisense probes were created with forward and reverse primers, respectively. Dissected CNS and alimentary canal (midgut, hindgut and foregut) tissues were used for the *in situ* hybridization. The tissues were fixed in 4% paraformaldehyde at 4 °C overnight, washed 3 times for 15 min with PBST (PBS and 0.2% Triton-X-100), treated with 10 µg/mL proteinase K (NEB) for 12 minutes, re-fixed in 4% paraformaldehyde for 15 min, and hybridized at 48 °C for 20–30 h. After hybridization, the tissues were washed with hybridization solution, blocked in 1% BSA (Bovine serum albumin), and incubated with anti-digoxigenin-alkaline phosphatase (Roche, 1:1000 dilution in 1% BSA) overnight at 4

°C. The tissues were then washed and developed using nitroblue tetrazolium salt/5-bromo-4-chloro-3-indoyl phosphate (NBT/BCIP, Roche) in the alkaline phosphatase buffer. Color development was stopped by repeated washes with PBS, and the tissues were subsequently mounted in 100% glycerol on glass slides.

2.5. Immunohistochemistry

The antibodies against TcOKA and TcOKB were raised in a rabbit and a chicken (Genescript, Nanjing, China), respectively. Slight modifications were applied for the antigenic peptide synthesis. NFGVLQLGGGYGVAC and CSLDRIGGGNLVamides, for TcOKA and TcOKB, respectively, were chemically synthesized and conjugated to keyhole limpet hemocyanin (KLH) for the cysteine residues tailed in the C- and N-termini of the respective peptides. The final bleed was used for affinity purification.

The last instar larvae were dissected in ice-cold phosphate-buffered saline (PBS: 137 mM NaCl, 1.45 mM NaH₂PO₄, 20.5 mM Na₂HPO₄, pH 7.2). The CNS and alimentary canal were fixed in Bouin's solution (37% formaldehyde and saturated solution of picric acid 1:3) at 4 °C overnight. The fixed samples were washed in PBS containing 1% Triton X-100 (PBST). The tissues were then preadsorbed with 5% normal goat serum (Sigma) in PBST for 10 minutes and subsequently incubated with anti-TcOKA (1:1000) and anti-TcOKB (1:500) antibodies for 2 days at 4 °C. After three washes with PBST (5 min each), the tissues were incubated overnight in goat anti-rabbit (conjugated with Alexa Fluor 647, Molecular Probes) and anti-chicken IgG antibody (conjugated with Alexa Fluor 488, Molecular Probes). The tissues were washed in PBST and mounted in glycerol containing 300 nM 4',6'-diamino-2-phenylindole (2 µg ml⁻¹; Sigma). Images were captured using a confocal microscope (Zeiss LSM 700). Schematic drawings were made in Adobe Photoshop 7.0 or Illustrator. The presented data (Fig. 4B) represent staining patterns reconstructed based on multiple samples as specified in the figure captions.

2.6. RNA interference

Primers (Table 1) tailed with T7 promoters on the 5' side were used to synthesize isoform-specific dsRNA of the same regions in the *in situ* probes, which target specific region for each isoform. The purified PCR products were utilized for dsRNA synthesis using a TranscriptAid T7 High Yield Transcription Kit (Thermo Scientific). A total of 150 ng dsRNA in approximately 100 nL was injected into the body cavity. The dsRNA injections were made at two different developmental stages, i.e., the 4th instar larval and early pupal (within 24 hours after pupation) stages. Seven days after larval injection, the larvae were subjected to the water knockout assay. The pupal injection was followed by a behavioral test that included mobility and death feigning assays. In these assays, 7–10-day old virgin adults were used. The deaths that occurred less than 5 days after injection were considered to be due to injection injury and excluded from the data analyses (less than 10%). Seven days after injection, 4 individuals injected with dsRNA from each treatment were pooled for the RT-PCR to assess the efficiency of the RNAi.

2.7. Behavioral analysis after RNAi

For the mobility assay, an arena with an oval-shaped cutout (1.5 x 5 cm) on a 3-mm thick aluminum plate was built as our mobility assay chamber (Kim et al.). The aluminum plate was placed on filter paper and covered with a glass plate. One beetle placed in the oval cutout was video recorded for 15 minutes beginning 30 seconds after the beetle was introduced. The speeds and turn angles in the arena were video recorded and analyzed with EthoVision XT 7 (Noldus Information Technology, Wageningen, Netherlands). Five individuals per treatment were examined. The statistical analyses were performed with ANOVA and Tukey HSD tests, and the level of significance was set at $P = 0.05$.

For the death feigning assay, each beetle was gently immobilized by adhering double-sided tape (3M Double coated urethane foam tape 4016 off-white, 3M, St. Paul, MN, USA) to its back. Death feigning was induced by stroking the ventral surface of the thoracic segment in the posterior to anterior direction using a soft plastic stick. Each touch was counted as one trial. The duration of the immobilization period following each touch was measured. Consecutive stimulations were repeated after the beetle recovered for a total of 11 trials or until the immobilization period was longer than 60 seconds. Leg movement of the beetle was the major criteria for quantifying death feigning. Nine seconds, which satisfied the $P = 0.05$ significance level for the Poisson distribution of the log-transformed durations of the death feigning of the non-injected controls over a total of 626 trials, was set as the threshold for the immobilization duration used to calculate the frequency of significantly longer death feigning durations.

Briefly, in the water knockout assay, ~4th instar larvae were submerged in ddH₂O for 2 minutes for complete knockout. Then, they were transferred onto a dry paper towel. The time required to recover from knockout was measured. All behavioral assays were performed between 1 and 7 PM.

3. Results

3.1. Orcokinin in *Tribolium*

We confirmed the presence of two alternatively spliced transcripts in *T. castaneum*, which were not included in the previous neuropeptide annotation (Li et al., 2008). The first two exons encode the first 30 amino acids and were used in both the *ok-a* and *ok-b* transcripts. The *ok-a* transcript encoded E3a to E6a (Fig. 1), resulting in the open reading frame (ORF) with 177 amino acid residues, while the *ok-b* transcript encoded the E3b ORF with 184 amino acid residues. The OK-A pre-propeptide contained a clear orcokinin homology (NFGVLQLGGGYGVA), with N-terminus R (Arg) and C-terminus KR (Lys-Arg) as the putative cleavage sites. OK-B pre-propeptide carried at least 10 isopeptides; 6 of these peptides contained a (S,G)VDPIDGDLamide, 3 contained a SLD(R,G)IGGGNLamide, and one contained a SVDPIDGDDLamide. The other two largely divergent (QWSRLFamide and LLDGYRRKHamide) peptides contained the canonical amidation signal GR (Gly-Arg) at their C-termini.

Expanded searches of the OK-A and OK-B sequences in NCBI database yielded a large number of genes carrying the alternatively spliced forms of OK-A and OK-B in insect

species. (Fig. 2). Interestingly, the categorical homology is extended to other invertebrates, crustaceans, and arachnids (Fig. 2). OK-A was generally characterized by N-terminus NFDEID and carried one or two Fs in the C-terminal region, with some exceptions. OK-B was generally characterized by a hydrophobic amino acid (I, L, or V) immediately followed by highly conserved D in the second or third amino acid of the N-terminus. A string of Gs (Gly-Gly-Gly) followed two amino acids later, with some variation to form the N-terminal consensus X(I,L,V)DXXGGG in general. The C-terminus often contained an H followed by a hydrophobic amino acid (i.e., L). The gene structure of orckinin in crustaceans, arachnids, and annelids commonly contains OK-B in the N-terminus of the pre-propeptide and is followed by multiple OK-As (denoted by lower case -a and -b to indicate variants in the same gene compared with capital -A and -B, which are used to indicate alternative splicing variants in Fig. 1). This pattern is similar to the 5' region of the alternatively spliced exon encoding OK-B in insects. The sequence homology further extends to Nematoda and Mollusca, but there were difficulties in categorizing the peptide as either the -A or -B form. Although the homologies were weak in the sequences of these taxa, the diagnostic amino acids, i.e., D as the 3rd and G as the 7th amino acid, FGF in nematodes and HGL in Mollusca in the C-terminal region, support the ancestral homology of orckinin (Fig. 2).

3.2. Isoform-specific quantitative reverse transcriptase-PCR (Q-RT-PCR)

The transcript levels of *Tcok-a* and *Tcok-b* were investigated with quantitative RT-PCR (Q-RT-PCR). Among the eight different examined stages, i.e., early embryonic (EE, <24 h), late embryonic (LE, >24 h), early larval (EL, <24 h post-hatching), late larval (LL, older than 5th instar including prepupae), early pupal (EP, <24 h post-pupation), late pupal (LP, >72 h post-pupation), early adult (EA, <24 h post-eclosion), and late adult (LA, one week old), the LL stage exhibited the highest levels of both the *Tcok-a* and *Tcok-b* transcripts (Fig. 3). The LL stage exhibited the greatest expression and was further investigated for tissue-specific expression patterns. Among the four different tissues examined, i.e., carcass, central nervous system (CNS), hindgut and midgut, the midgut exhibited the highest levels of both *Tcok-a* and *Tcok-b* transcripts, while the CNS also exhibited low levels of the *Tcok-a* transcript. The *Tcok-b* transcript in the CNS was minimally present in the LL stage. Interestingly, the levels of the *Tcok-a* and *Tcok-b* transcripts were highly correlated across the stages.

3.3. In situ hybridization and immunohistochemistry

In situ hybridization was performed in the late larval stage, which was the stage that exhibited the highest levels of both the *Tcok-a* and *Tcok-b* transcripts in the Q-RT-PCR analyses. Exon-specific antisense probes (exons 3a to 5a for *ok-a* and exons 3b for *ok-b*, Fig. 1) revealed identical staining patterns in the CNS (Fig. 4) and midgut (Fig. 5), and the hindgut and foregut were negative. Six pairs of cells in the brain were positive, but no other positive cells were found in the thoracic or abdominal ganglia in the CNS for either the *ok-a* or *ok-b* probes. Sense probes, the negative controls, yielded no specific staining.

Immunohistochemistry with antibodies specific for each OK-A and OK-B in the larval stage revealed a pattern similar to the results of the *in situ* hybridization. However, the individual variations were large in terms of obtaining consistent staining patterns in the 4 biological

replications (~10 individual in each replication). Based on all of these data, we concluded that five pairs of immunoreactive cells were identical to the cells revealed by *in situ* hybridization, whereas one pair was positive only by *in situ* hybridization, and another pair of cells was only identified by immunohistochemistry (Fig. 4B). We found that the positive cells were positive for both OK-A and OK-B based on the double staining and based on the locations of the cells in each separate staining. There were no positive cells in any other ganglia. The patterns of varicosities indicated that the projections were within the central nervous system and included the varicosities in the subesophageal ganglion and the first segment of the thoracic ganglion, which were likely projections originating from the brain cells. In the adult brain, positive cell bodies were rarely visible in the PLM, PMN, and DLM (Fig. 4 caption for abbreviations), while axonal projections in the dorsal brain were commonly found in different individuals.

The staining patterns in the alimentary canal robustly indicated the slender enteroendocrine cells over the entire length of midgut but not in the foregut or hindgut (Fig. 5). Adult enteroendocrine cells appear smaller than those in the larval stage. The colocalization of OK-A and OK-B in the same cells was apparent in the double staining and also based on the locations of the cells in each separate staining. However, we found that the subcellular localizations of the staining patterns suggested that the immunoreactivities were possibly compartmentalized in different region of the cells, presumably in different pools of secretory vesicles. For example, in the cells with obvious subcellular compartmentalization, TcOK-B (green fluorescence) was often observed in the hemolymph side, while TcOK-A (red fluorescence) was typically present in the luminal side of the cells. *In situ* hybridization also revealed identical patterns of slender endocrine cells in the midgut. These specificities of *in situ* hybridization and immunohistochemistry were confirmed by the appropriate pre-immune serums and RNAi, which abolished the staining (Supplementary Fig. S1).

3.4. RNAi

We found that the injection of *T. castaneum* larvae or early pupae with ds*Tcok-a*, ds*Tcok-b*, or mixture of the two did not cause significant mortality or observable morphological or behavioral differences compared with the uninjected or buffer-injected controls, while the suppressions of the target transcripts were confirmed by Q-RT-PCR. For example, the walking speeds of the adults measured in a small arena exhibited no difference between the control and RNAi treated insects (Supplementary Fig. S2). Serendipitously, we found that the adults treated with dsRNA displayed a high susceptibility to external stimuli in that they exhibited more frequent and longer death feigning. Death feigning in *T. castaneum* has previously been described as an inheritable behavior for avoiding predation (Miyatake et al., 2004).

To quantify the death feigning response, we measured the duration of death feigning induced by brushing the thoracic ventral midlines of beetles that were immobilized with double-sided tape on their backs by a plastic brush. Death feigning was defined by the lack of movement of the appendages, including the legs. A stroke of the brush was given immediately after the beetle recovered from the death feigning, and this process was repeated 10 times. The data were analyzed in terms of the frequency of strokes that induced

death feigning with the duration longer than 9 seconds, which was for $P = 0.05$ in the Poisson distribution of the log-transformed death feigning duration in the control (626 trials). We found that the treatments with *Tcok* dsRNA increased the death feigning duration. More than 50% strokes in the dsRNA-treated beetles, but less than 20% strokes in the controls induced 9-second or longer death feigning (Fig. 6A).

We questioned whether OKs were also involved in the awakening response in the larval stage. We developed a new bioassay for investigating the larval stage, which does not induce death feigning behavior in response to mechanical stimuli. We developed an assay that measured the recovery time after the knockout of the larva by submersion into water. We submerged the larva in water for 2 min and measured the time required for awakening after the larvae were transferred to the surface of dry paper towel. The treatments with *dsTcok-a*, *-b*, and both resulted in significantly delayed awakening times following the knockout (Fig. 6B).

4. Discussion

The products of alternatively spliced forms of orcokinin gene, OK-A and OK-B, have been previously described and commonly identified in a number of insect species (Sterkel et al., 2012). The alternative forms are distinguished by their sequence motifs, which stem from ancestral gene structures in Crustacea, Arachnida, and Annelida that encode the sequence motifs of both OK-A and OK-B (orcomyotropin and orcokinin in Crustacea) in a single transcript, without evidence of alternative splicing. Therefore, the parsimonious interpretation of the evolutionary process responsible for the alternatively spliced exons is that the evolution of two different sequence motifs was first and followed by the splitting of the exon into two alternative exons. This interpretation contrasts with the situation of alternative-exon formation by exon duplication and subsequent diversifying evolution (i.e., exon shuffling, (Keren et al., 2010). However, the earliest appearance of two different orcokinin sequence motifs is not clear, because Nematoda, which is known to be a closer sibling of Insecta than Annelida, has an OK sequence motif that is not distinguished by isoforms (Fig. 2).

TcOK-A sequence is strikingly different from OK-As of other arthropods and highly similar to OK-B sequence by carrying GGG motif in the middle of the predicted mature peptide, although the phylogeny of the pre-propeptide supports that the TcOK-A belong to the orthology group of OK (data not shown). We speculate that this unusual pattern of evolution in TcOK occurred as a consequence of pleiotropism in the ligand-receptor interactions, in which cross-activity of ligands to receptors may be favored in the specific evolutionary lineage (Jiang et al., 2014). Although the receptors for each peptide have not yet been identified, this case offers an opportunity to investigate the role of pleiotropism in ligand-receptor evolution.

In this study, we aimed to distinguish the expression patterns of OK-A and OK-B using exon-specific probes in *in situ* hybridization and isoform-specific antibodies raised in chickens and rabbits, because such a distinction may be informative regarding functional diversification. Surprisingly, we were unable to identify differential patterns of OK-A and

OK-B expression; rather, we observed identical cellular expression patterns in the brain and the enteroendocrine cells over the entire midgut. Additionally, stage-specific Q-RT-PCR revealed similar temporal expression patterns. Our findings in *T. castaneum* contrasted with those in *D. melanogaster* (Chen et al.; Veenstra and Ida, 2014) and *R. prolixus* (Sterkel et al., 2012). The expression patterns of the isoforms have recently been studied in the highly derived species *D. melanogaster*. This study clearly reveals different expressions of OK-A and OK-B in this species; OK-A is expressed in the cells of the central nervous system including the cells in segmental ganglia, and OK-B is expressed in the enteroendocrine cells (Chen et al.). RT-PCR in *R. prolixus* revealed that OK-A is exclusively expressed in the nervous system, while OK-B is expressed both in the nervous system and the anterior midgut (Sterkel et al., 2012). In *B. mori*, numerous cells in the brain, ganglia, and midgut produce positive *in situ* hybridization results (Yamanaka et al., 2011). The probe for the *in situ* hybridization in the *B. mori* OK-A study included the 5' common exon, which cannot discriminate between the isoforms; thus, the expression patterns of the exon-specific isoforms are unknown. In a crustacean species, OK-A and OK-B (orcokinin and orcomyotropin, respectively, Dirksen et al., 2000), which are encoded by a single transcript, are both found in the stomatogastric nervous system and pericardial organs based on a proteomics study (Dirksen et al., 2000). Interestingly, the OK-A and OK-B immunoreactivities in *T. castaneum* were found to differ in terms of subcellular localization in the enteroendocrine cells (Fig. 5E), which supports the possibility that separate pools of secretory vesicles contain OK-A and OK-B, although this hypothesis requires further testing with an ultrastructural study.

We investigated the functions of the OK-A and OK-B isoforms in *T. castaneum* by RNAi. Suppression of the expressions of the target transcript was apparent as shown in Supplementary Fig. S1, like the RNAi of other neuropeptide transcripts in previous studies (Aikins et al., 2008; Arakane et al., 2008). Our initial trials with RNAi suppression of the orcokinins alone or in combination did not reveal obvious phenotypes in terms of survivorship or morphological deficiencies in laboratory conditions. During the investigation, we noticed that the beetles treated with dsRNA for orcokinins displayed more frequent death feigning. It was obvious that either RNAi for OK-A and OK-B alone or in combination resulted in longer durations of death feigning in response to the stimulus of brushing the central midline of the ventral thorax, although this assay was unable to determine whether the RNAi increased the sensitivity to the stimuli, delayed the recovery, or both. Previous studies of death feigning in *T. castaneum* have shown that the length of death feigning is a heritable characteristic (Miyatake et al., 2004). The involvement of dopamine in this heritable characteristic has also been suggested (Miyatake et al., 2008).

The physiological function of OK-A is an important factor in the light entrainment pathways that are involved in circadian rhythms, as indicated by the finding that the injection of OK-A caused circadian phase shifts and the expression pattern of OK-A included the accessory medulla in *L. maderae* (Hofer and Homberg, 2006b). In our study, the death feigning phenotype of the RNAi-treated beetles, together with the larval RNAi phenotype (i.e., the delayed recovery from knockout induced by water submersion), suggests that the orcokinins function in “awakening”. The awakening activity appears to be distinct from “arousal”

status, because the adults treated with RNAi did not exhibit differences in normal activity in our observations of walking speed and turn angles in a small arena over 15 min (Supplementary Fig. S2). The “awakening” function of orckinins in this study indicated that orckinins are involved in behavioral responses to stress in *T. castaneum*.

In conclusion, the orckinins in *T. castaneum* exhibit largely divergent amino acid sequences relative to the orckinins of other insect species, but they retain similar gene structures in terms of the alternatively spliced forms OK-A and OK-B. The “awakening” activities of both OK-A and OK-B in *T. castaneum* observed in the behavioral bioassays. Further researches on identification and characterization of the orckinin receptors, and neurohormonal regulation of the circadian rhythms in *T. castaneum* will shed the light on the functions of TcOKs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Alternatively spliced isoforms of orcokinin, OK-A and OK-B, were identified in *T. castaneum*.
- Co-expression of OK-A and OK-B in the brain and midgut cells were revealed in this species.
- RNAi in conjunction with novel behavioral assays suggest “awakening” activity of orcokinins.

>TcOK-A

MRFVTVAIVIFVAAAATEAAPKPAGLSGYSPYEEVIGQKTIR**NFGVL**
QLGGGYGVAKRFAQPGKYDTKTEKHRGPLNGLIPGGAFGRAARSCL
 KTNCLRLLKGQMDAKIHPYFSFDSDRQATKPGIYKTYQLLEME

>TcOK-B

MRFVTVAIVIFVAAAATEAAPKPAGLSGYSRRQWSRLFGR**SVDPIDG**
DLIGR**SVDPIDGDLI**GR**SVDPIDGDLI**GR**SLDRIGGGNLV**GR**SVDPID**
DGDLIGR**SVDPIDGDDLI**GR**SLDRIGGGNLV**GR**SVDPIDGDLI**GR**SL**
DGIGGGNLVGR**GVDPIDGDLI**GR**SLDIKRLLDGYRRKHNGRSL**

Fig. 1.

Gene structures and deduced amino acid sequences of the orcokinin transcripts in *Tribolium castaneum*. The underlined italic letters indicate the putative signal peptides, the underlined basic amino acids (K or R) and Gly (G) at the ends of putative mature peptides (bold) indicate the canonical cleavage and amidation signals.

Orcokinin A

	<i>Drosophila melanogaster-A</i>	1x	NFDE DKASASFS LNQLV
	<i>Manduca sexta-A</i>	2x	NFDE DRSS
Insecta	<i>Tribolium castaneum-A</i>	1x	NFGVLQLGGGYGVA
	<i>Apis mellifera-A</i>	2x	N DE DR F
	<i>Acyrtosiphon pisum-A</i>	3x	NFDE DRS F FV
Crustacea	<i>Amphibalanus amphitrite-a</i>	3x	NFDE DRSGF GF
	<i>Daphnia pulex-a</i>	2x	NLDE DRS FG F
	<i>Homarus americanus-a</i>	8x	NFDE DRSGF GF
Arachnida	<i>Stegodyphus mimosarum1-a</i>	3x	NFDE DR GF
	<i>Stegodyphus mimosarum2-a</i>	13x	NFDE DRAGF -GKR
Anneida	<i>Platynereis dumerilii-a</i>	20x	FDS GH SN FAGLD

Orcokinin B

	<i>D. melanogaster-B</i>	1x	GLDS GGGHL
	<i>M. sexta-B</i>	12x	NLRSL GG -GR
Insecta	<i>T. castaneum-B</i>	10x	SYD P DGD L -GR
	<i>A. mellifera-B</i>	4x	NLD G GNL
	<i>A. pisum-B</i>	5x	S SLDRLNGGH M A
Crustacea	<i>Amphibalanus amphitrite-b</i>	5x	G DSLGGGHLL
	<i>Daphnia pulex-b</i>	1x	S L DS G S
	<i>Homarus americanus-b</i>	3x	FDAFTTGFGHN
Arachnida	<i>Stegodyphus mimosarum1-b</i>	1x	SLDRLGGGEL R
	<i>Stegodyphus mimosarum2-b</i>	1x	... LDK GGGMVL
Anneida	<i>Platynereis dumerilii-b</i>	5x	QLD LGG A P L Y -GR

Putative ancestral orcokinin

Nematoda	<i>Ascaris suum</i>	14x	ALD L EG GF
	<i>Caenorhabditis elegans</i>	12x	ALB L DG GF
Mollusca	<i>Tritonia diomedea</i>	11x	R YDQ TGLHGLSGFA
	<i>Lymnaea stagnalis</i>	16x	PFDS SGSHGLSGFA

Fig. 2.

Putative mature peptide sequences of orcokinin A and orcokinin B. The amino acid sequences are sequence logos for the putative mature peptides that are repeated in the pre-propeptide of each species. The species names followed by -A or -B denote alternatively spliced isoforms, and -a and -b denote variants within the same transcript.

Fig. 3.

Transcript levels of *Tcok-a* and *Tcok-b* measured by Q-RT-PCR. (A) Tissue-specific patterns: carcass, CNS (central nervous system), MG (midgut), and HG (hindgut). The transcript levels were standardized by setting CNS to 1. (B) Developmental patterns: EE (early embryonic), LE (later egg), EL (early larvae), LL (late larvae), EP (early pupae), LP (late pupae), EA (early adult), and LA (late adult, see more details in text). The transcript levels were standardized by setting EE to 1. The relative mRNA levels were all normalized to the reference gene *ribosomal protein S3* (*rpS3*).

Fig. 4.

Expression patterns of TcOK-A and TcOK-B in the brain of *T. castaneum*. (A) *In situ* hybridization for *Tcok-a*. The result of *Tcok-b* is similar to that of *Tcok-a* based on the composite image of more than 20 samples, though individual variation was high (data not shown). (B) Diagram showing the consolidated view of the *in situ* hybridization and immunohistochemistry: DLN, deutocerebral lateral neuron; DMN deutocerebral median neuron; PMN, protocerebral median neuron; PLN, protocerebral lateral neuron; PSN, protocerebral superior neuron. The cells with red color (PSN2) were found only by *in situ* hybridization and not by immunohistochemistry. The cells with blue color (PMN2) were found only by immunohistochemistry and not by *in situ* hybridization. (C, D, E) Larval

brains stained with anti-TcOK-A (red) and anti-TcOK-B (green) and the merged images. (F, G, H) Adult brain stained with anti-TcOK-A (red) and anti-TcOK-B (green) and the merged images. Arrows in (E) indicate the locations of the cell bodies. Scale bar = 50 μm .

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Fig. 5.
Expression patterns of TcOK-A (red) and TcOK-B (green) in the midgut of *T. castaneum*. (A) Merged confocal microscopy image collapsed over a total of 50 μm in the Z-stack. (B) Magnified view of the larval midgut layer. On this left is the lumen of the gut. Blue indicates nuclei stained with DAPI. (C) Magnified view of an adult midgut layer. The left upper region is the lumen of the gut. Small enteroendocrine cells are stained. (D) Top view of a merged and magnified image of the inset in (A). (E) Magnified view of the inset in (B) showing the subcellular staining patterns of TcOK-A and TcOK-B. (F, G) *In situ* hybridizations of *Tcok-a* and *Tcok-b*. Scale bar = 10 μm

Fig. 6. Behavioral assays showing the increased death feigning rate in the adult stage (A) and the longer recovery time following knockout induced by water submergence in the larval stage (B).

Table 1

Primers used in this study

Experiments	Genes	
	<i>Tcok-a</i>	<i>Tcok-b</i>
Cloning	R: CATCTAGCTACACAAGTCCAAC	F: AATCATGCGTTTTTGTGACC
	F: CGAAGGGACCTCTCAATG	R: TAGTTACGTTTATTGGATTATTG
Q-RT-PCR	R: GTTGCTTGTCTATCGCTGTCA	F: AGGAGTCTGGACGGGATAGG
	F: taatacgactactatagggCTTACGAGGAGGTGATTGG	R: ACCATTGTGTTTTTCGTCTGTATC
dsRNA synthesis	R: taatacgactactatagggTTACTCCATTTCCTCAATAATTG	F: taatacgactactatagggAATGGAGCCGGTTGTTTG
	Sense: CTTACGAGGAGGTGATTGG	R: taatacgactactatagggTAAAGTGATCGACCATTTGTG
<i>In situ</i> hybridization	Anti-sense: AAGTCAAAATTACTCCATTTC	Sense: AATGGAGCCGGTTGTTTG
		Anti-sense: CAATACAAAGTAATTAAAGTGATCG

Note: F means forward primers; R means reverse primers. Letters in lowercase are the T7 promoters for dsRNA synthesis. Sense and anti-sense mean the primers used for sense and anti-sense probes synthesis in *in situ* hybridization, respectively.