Insect Biochemistry and Molecular Biology 38 (2008) 740–748

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

Vasopressin-like peptide and its receptor function in an indirect diuretic signaling pathway in the red flour beetle

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ARTICLE INFO

Article history:
Received 24 April 2008
Accepted 30 April 2008

Keywords:
Diuresis
Coleoptera
Beetle
Cryptonephridial complex
Neuropeptide
Evolution

ABSTRACT

The insect arginine vasopressin-like (AVPL) peptide is of special interest because of its potential function in the regulation of diuresis. Genome sequences of the red flour beetle *Tribolium castaneum* yielded the genes encoding AVPL and AVPL receptor, whereas the homologous sequences are absent in the genomes of the fruitfly, malaria mosquito, silkworm, and honeybee, although a recent genome sequence of the jewel wasp revealed an AVPL sequence. The *Tribolium* receptor for the AVPL, the first such receptor identified in any insect, was expressed in a reporter system, and showed a strong response ($EC_{50} = 1.5$ nM) to AVPL F1, the monomeric form having an intramolecular disulfide bond. In addition to identifying the AVPL receptor, we have demonstrated that it has *in vivo* diuretic activity, but that it has no direct effect on Malpighian tubules. However, when the central nervous system plus corpora cardiaca and corpora allata are incubated along with the peptide and Malpighian tubules, the latter are stimulated by the AVPL peptide, suggesting it acts indirectly. Summing up all the results from this study, we conclude that AVPL functions as a monomer in *Tribolium*, indirectly stimulating the Malpighian tubules through the central nervous system including the endocrine organs corpora cardiaca and corpora allata. RNA interference in the late larval stages successfully suppressed mRNA levels of *avpl* and *avpl* receptor, but with no mortality or abnormal phenotype, implying that the AVPL signaling pathway may have been near-dispensable in the early lineage of holometabolous insects.

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1. Introduction

Arginine vasopressin (AVP) is a highly conserved nonapeptide, widespread in the metazoa. Related neuropeptides include oxytocin (OT) in mammals and isotocin and vasotocin in other vertebrates. The divergence of AVP and OT appears to have occurred early in the vertebrate lineage. In invertebrates, similar peptides have been described in the earthworm (Oumi et al., 1994), snail (van Kesteren et al., 1992), octopus, (Takuwa-Kuroda et al., 2003), and the locust (Remy et al., 1979). In mammalian species, AVP and OT exert a variety of functions including anti-diuresis and blood pressure regulation by AVP, and smooth muscle contraction during birth, lactation, and maternal behavior by OT. This group of peptides has been reported to have similar functions in invertebrates, including induction of egg-laying behavior and mucus secretion in annelids (Oumi et al., 1996), induction of smooth muscle contraction in octopus (Takuwa-Kuroda et al., 2003), and stimulation of excretion (diuresis) in the Malpighian tubules (MT) of the locust (Proux and Herault, 1988).

Earlier studies of the insect AVP-like (AVPL) peptide were motivated by the discovery of cells in the subesophageal ganglion of *Locust migratoria* that were immunoreactive toward an antibody raised against mammalian AVP (Remy et al., 1979). This observation was later extended to other locust species, cockroaches and mantids (Davis and Hildebrand, 1992; Tyrer et al., 1993). The discovery of AVP immunoreactivity in locust led to the isolation and identification of the AVPL peptide and to studies of its diuretic function in locust MT (Proux et al., 1987; Schooley et al., 1987). Proux et al. identified the presence of two isoforms of the peptide (Fig. 1), a monomeric form (F1), and an antiparallel homodimer (F2) in locust ganglia, but not a parallel homodimer (D2). Of the three isoforms of AVPL, F2 was found to be the most active for increasing the secretion of locust MT (Proux et al., 1988). However, the finding of diuretic activity of F2 and F1 was not confirmed in an independent investigation (Coast et al., 1993).

The genome sequences of holometabolous insects such as the fruitfly (*Drosophila melanogaster*), malaria mosquito (*Anopheles gambiae*), and honeybee (*Apis melifera*), all lack the gene for AVPL,

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doi:10.1016/j.ibmb.2008.04.006

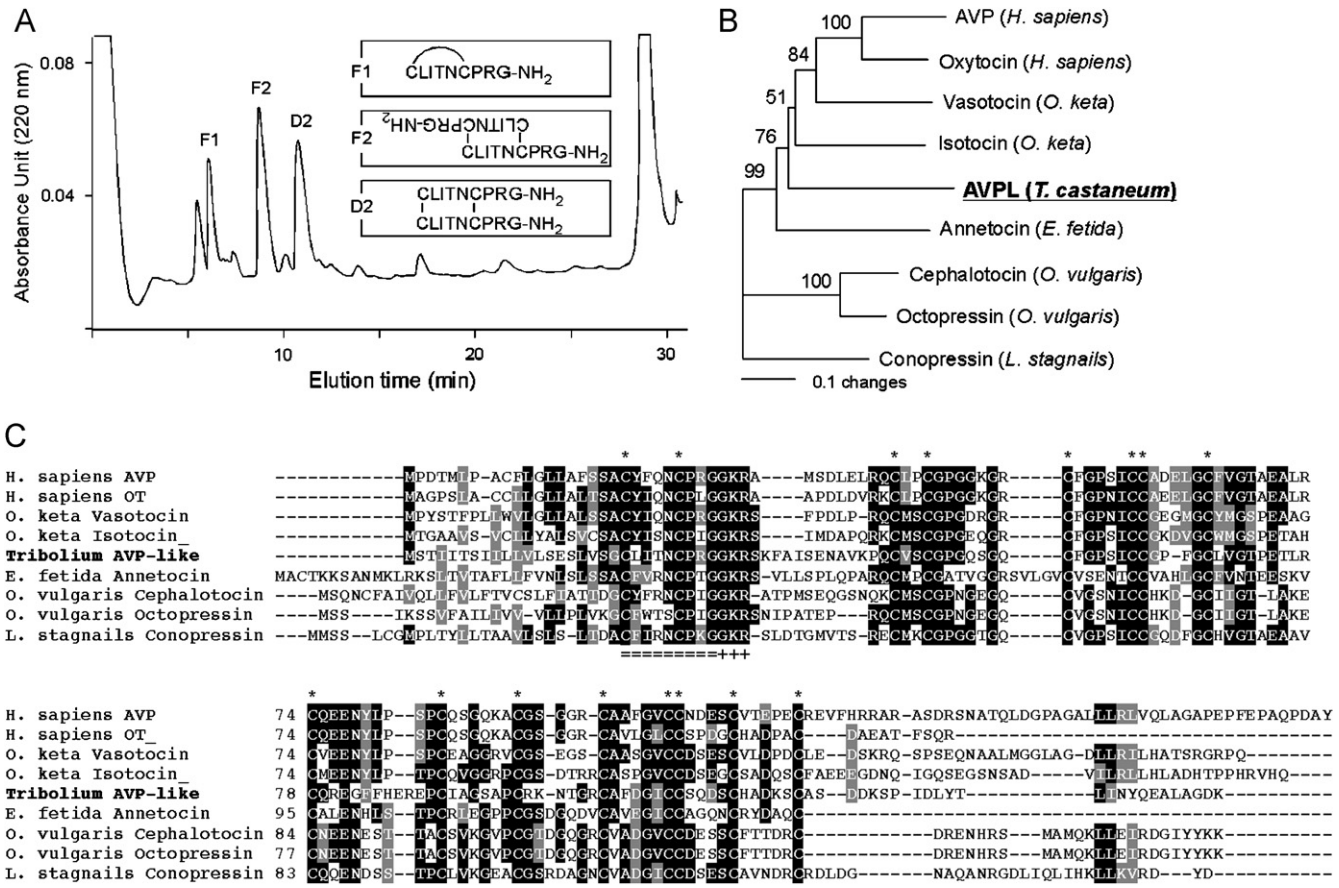

Fig. 1. (A) Analysis of arginine vasopressin-like peptide after base-catalyzed non-specific synthesis of the antiparallel dimer F2 and parallel dimer D2 from the monomeric F1. Absorbance was monitored at 220 nm. Slower eluting impurities include trimers and tetramers of F1. (B) The phylogenetic relationship of the pre-propeptide sequences of AVPL-related peptides including vertebrate AVP and OT and invertebrate AVP. The tree was constructed by a neighbor-joining method. Numbers at the nodes are for percent supports in 1000 bootstrapping. A parsimonious tree provided the same tree topology. (C) Sequence alignment for AVP-related sequences. Inverted letters with gray background are similar, while black indicates identical amino acids in the sequence alignment with the 50% majority rule. Conserved cysteines are marked with stars (*) above the alignment. The mature AVP peptide is marked with double underline (=) and followed by canonical amidation and dibasic cleavage sites GKR (+ marks).

except a recent genome sequence of a jewel wasp (*Nasonia vitripennis*) contains a putative *avpl* gene. Likewise, there are no representatives of the orthologous group of AVP/OT receptors in these insect genomes (Park et al., 2002) with the exception of the jewel wasp. Our analysis of the genome sequence of the red flour beetle *Tribolium castaneum* has shed new light on the AVPL signaling pathway by identifying the genes encoding AVPL and AVPL receptor (AVPL-R). We describe the *avpl* and the *avpl-r* genes and the indirect diuretic function of AVPL in this species.

2. Materials and methods

2.1. Gene cloning and sequence analysis

The AVPL sequence described in the locust (Proux et al., 1987) was identified in the *Tribolium* genome sequence. The predicted coding regions for each AVPL and its receptor were amplified from a cDNA library (TL; *Tribolium* mixed stages of whole larvae), by the polymerase chain reaction (PCR) using internal pairs of primers and rapid amplification of cDNA ends (RACE). Subsequently, we also took advantage of available expressed sequence tag (EST) clones containing the AVPL receptor (Park et al., 2008).

Phylogenies of each conceptually translated *avpl* and *avpl-r* sequences were made based on the sequences aligned using CLUSTAL W (Thompson et al., 1994). The aligned sequences were used for the construction of a phylogenetic tree in PAUP 4b.2 (Swofford, 2000) by using neighbor-joining or parsimonious method with 1000 bootstrapping.

2.2. AVPL peptides

To make the dimers F2 and D2 from F1, 25.1 mg of F1 peptide was dissolved in 4 ml water and 0.8 ml of 6% aqueous triethylamine. The solution was vortexed and allowed to stand at room temperature for 4 h, then taken to dryness in a vacuum centrifuge. The scrambled peptides were separated with a ThermoSeparations P4000 preparative HPLC equipped with a 250 × 26 mm ModCol wide pore C_{18} column eluted at a flow rate of 20 ml/min using a multi-segment linear gradient. The peptides eluted in a segment starting at 15% of 95% ethanol/0.1% trifluoroacetic acid (TFA) increasing to 33% ethanol/0.1% TFA in 50 min.

Fractions were analyzed by MALDI-TOF MS using an ABI Model 4700. Retention times of samples were compared to those of authentic standards of dimers F2 (antiparallel) and D2 (parallel) by analytical HPLC. A Hewlett-Packard Model 1050 was equipped with a 3.5-μm Zorbax 300SB-C_8 column, 100 × 3 mm. Peptides were eluted with a multi-segment linear gradient starting at 2% CH_3CN/0.1% aqueous TFA, increasing to 14% CH_3CN at 1 min, then increasing to 23% CH_3CN at 26 min, and finally increasing to 60% CH_3CN at 36 min (Fig. 1A).

2.3. Immunohistochemistry and in situ hybridization

The central nervous system (CNS) was dissected in PBS and fixed overnight in Bouin at 4 °C. The tissues were then washed 3 × 15 min in PBST (PBS with 0.1% Triton X-100) and incubated with rabbit anti-vasopressin (1:200, Calbiochem, Cat No. PC234L) in PBST at 4 °C overnight. The sample was washed 3 × 15 min with PBST and incubated with a goat anti-rabbit conjugated overnight with Cy3 (Jackson Immuno Research, West Glove, Pensylvania) in PBST at 4 °C. The tissues were washed with PBST and mounted on a glass slide for observation using a confocal microscope LSM510.

For *in situ* hybridization, the Bouin-fixed tissues were treated with 10 μg/ml proteinase K for 10 min at room temperature. The reaction was terminated by the addition of PBST–glycine (2 mg/ml glycine) for 5 min. Samples were fixed again in 4% paraformaldehyde at RT for 1 h and washed with PBST for 5 min. Prehybridization for 10–30 min was followed by hybridization with a solution (50% formamide, 5xSSC, 50 μg/ml heparin, 0.1% Tween 20, and 100 μg/ml salmon sperm DNA) containing the digoxigenin labeled probe (Roche Molecular Biochemicals) for 24 h. Single-stranded 583 nt DNA probes (open reading frame (ORF) 1-538) were generated by asymmetric PCR for sense (negative control) or anti-sense and boiled for 40–60 min prior to hybridization. Tyramine-based fluorescence detection was done by using anti-digoxigenin-POD and Alexa Fluor-488-labeled tyramide (Invitrogen, Carlsbad, CA).

2.4. Real-time quantitative RT-PCR

Total RNA from a pool of two individuals of different stages or different tissues was prepared using TRIzol reagent (Invitrogen). The stages tested were egg, early larval (EL, < 24 h post-hatching), late larval (LL, older than fifth instar including prepupae), early pupal (EP, < 24 h post pupation), late pupal (LP, > 24 h post pupation), early adult (EA, < 24 h post eclosion), and late adult (LA, 1-week old). Tissue-specificity of expression was examined by quantitative PCR using SYBR premix Ex taq (Takara Bio., USA) in the CNS including all the ganglia and brain, MT, hindgut, and carcass excluding the aforementioned tissues. The amplicon for *avpl* was 108 bp (1–108 of ORF) and for *avpl-r* was 297 bp (878–1175 bp). The fold-differences of the target molecule were standardized compared with the control gene ribosomal protein S3 (RP3, GenBank accession number CB335975), using the ΔΔCT method (Livak and Schmittegen, 2001).

2.5. Functional assays of AVPL receptor

The AVPL receptor variants were cloned in the plasmids pCMV.SPORT6.1 or pXOON (Jespersen et al., 2002) carrying the cytomegalovirus (CMV) promoter for expression in mammalian cell lines. Transient expression of the GPCR clones was accomplished in the CHO-WTA11 cell line (Euroscreen) stably expressing the luminescent reporter protein apoaequorin (Rizzuto et al., 1992). Cells were grown in Ham's DMEM-F12 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml fungizone, 250 μg/ml Zeocin (Invitrogen). Transfection was performed using FuGene6 (Roche) according to the manufacturer's protocol at a ratio of DNA to FuGene6 of 3:1. Cell suspensions were loaded with coelenterazine 3 h (Invitrogen) before testing according to a previously developed protocol (Park et al., 2003; Poul et al., 2002).

Luminescence assays were performed in opaque 96 well microplates (Corning) using an Orion microplate luminometer (Berthold Detection Systems). Ligands being tested were plated in each well of the 96-well plate. Following the injection of cells into a well, the changes in luminescence were monitored for 20 s. The luminescent response was integrated over time and normalized to the largest positive control response in each plate and to the background values obtained from negative controls (buffer only).

2.6. In vivo excretion assay

Excretions from individual insects were detected using a modified system originally developed by Coast (2004). Constant velocity, dry air (~3% relative humidity (RH), 100 ml/min) was passed through a chamber containing a single adult *Tribolium*. Insect excretion, if it occurred, was instantly evaporated in the dry airflow and detected by the flow-through humidity analyzer RH-300 (Sable Systems, Henderson, NV). The saline used in the study was originally developed for *T. molitor* and contained (in mM) 90 NaCl, 50 KCl, 5 $MgCl_2$, 2 $CaCl_2$, 6 $NaHCO_3$, 4 NaH_2PO_4, 10 glycine, 10 proline, 6 histidine, 10 serine, 8 glutamine, 50 glucose, adjusted to pH 7.0 with NaOH (Nicolson, 1992).

Continuous 15-min recordings were made at a 60-Hz sampling rate after the treatment. All experiments used 1–4 week-old adults, and were performed at room temperature, which varied between 22 and 25 °C, in the months between July and October 2006.

2.7. MT secretion assay

To test the direct effect of AVPL on the secretory organ MT, we used isolated MT of *Tenebrio molitor*. The MT of larval *T. molitor* were dissected out and temporarily placed in a 100 μl drop of saline for the secretion assay. A single, isolated tubule was incubated in a 20 μl drop of saline that was covered with heavy mineral oil on a Sylgard-dish. The secretion forming a drop on the opening was withdrawn every 15 min using a Nanoinject 2000, which digitally measured the volume, with the smallest unit of measure being 1.15 nl. The tubules were preincubated for 20 min in the saline, and the secretion was measured for two consecutive 15 min intervals before and after the treatment. Data were retained only for those tubules shown to have stable secretion rates showing less than 25% change between the first and the second 15 min intervals. The peptides were tested at a concentration of 1 μM.

2.8. RNAi targeting avpl and avpl-r

DNA templates for double-stranded RNA (dsRNA) synthesis were prepared by PCR with T7-tailed primers based on *avpl* or *avpl-r*-specific sequences. The dsRNA for *avpl* is 399 bp in length (ORF 1–399 bp) and for *avpl* receptor is 486 bp in length (688–1156 bp, Fig. 2). dsRNA was synthesized using the MEGAscript RNAi Kit (Ambion, Inc. Austin, TX, USA) according to the manufacturer's protocol. A total of 200 nl of dsRNA was injected at a concentration of 0.6 μg/μl in 0.1 mM sodium phosphate, pH 7, containing 5 mM KCl. Penultimate-instar larvae were injected through the abdomen. After injection, larvae were allowed to rest in Petri dishes for 1 h, then returned to the incubator in Petri dishes with flour/yeast substrate and monitored daily for mortality, developmental rate, and for abnormal behavior. RT-PCR to confirm the suppression of target gene expression was conducted in 2 day old adults (10–15 days after dsRNA injection).

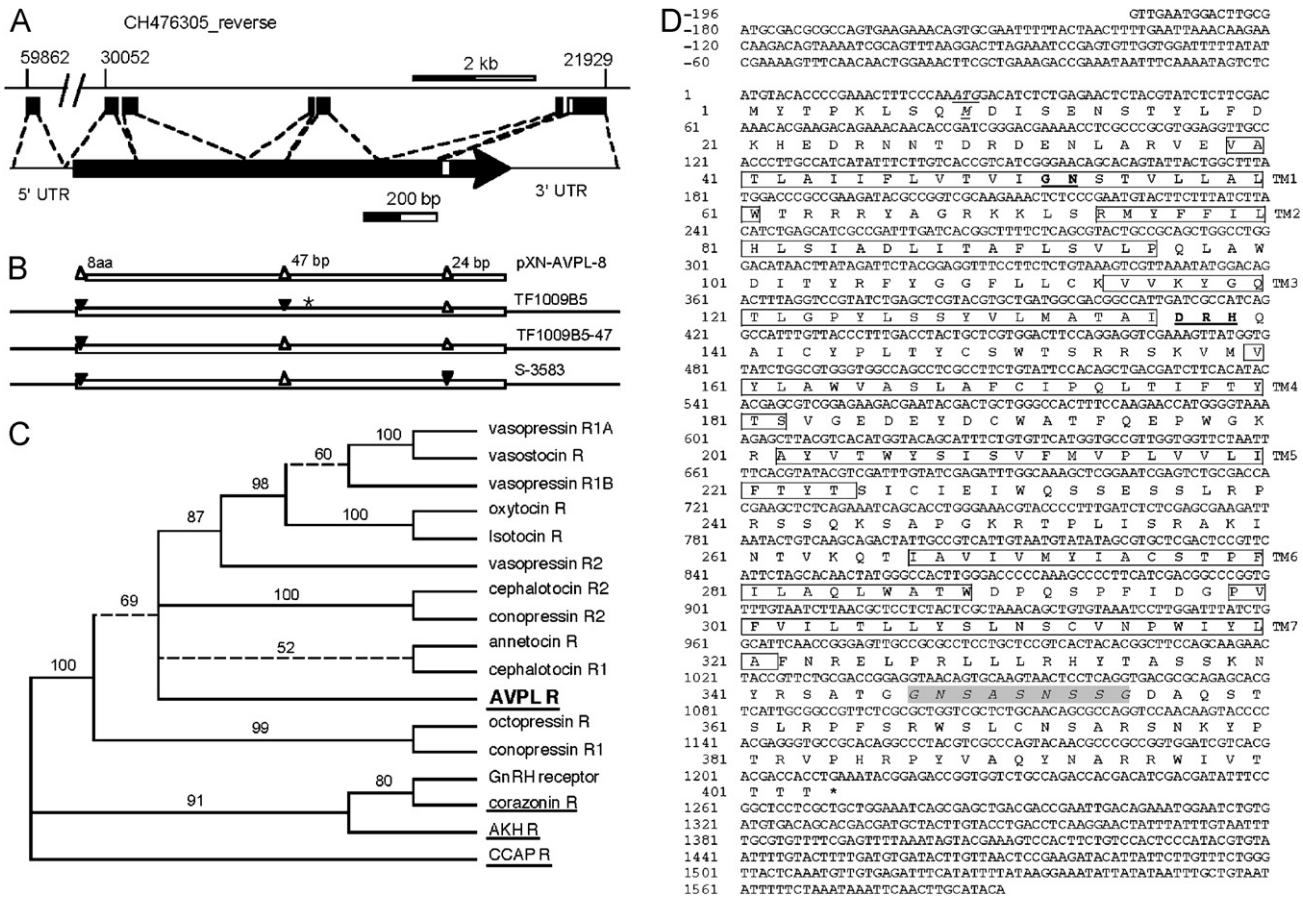

Fig. 2. (A) Gene structure of the *Tribolium* AVPL receptor. (B) Diagram showing the receptor variants cloned in this study. Empty triangle or inverted filled triangles indicates absence or presence of specific fragments, respectively, in the receptor variants cloned. (C) Phylogenetic relationship with other members of the AVP receptor group. Dotted branches are for the ones having bootstrapping supports lower than 70% in 1000 replications. Insect receptors are underlined. (D) *Tribolium* AVPL receptor sequence and conceptual translation. Transmembrane segments predicted by TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) are boxed in the translation. TM3 was manually adjusted for an extracellular cystine bond and for a cytoplasmic face for DRH. A second methionine, which was used in the clone pXN-AVPL-8 as translation initiation site, is underlined with italic in (B). The sequence conserved in the cytoplasmic surface of Family A G protein-coupled receptor GN and DRH (the variation of DRY) are bold and underlined (37, 38). Eight amino acids encoded by a 24-bp inclusive-exon (B) by an alternative splicing acceptor site are grayed.

3. Results

3.1. AVPL and AVPL receptor

The *avpl* gene is located in linkage group 8 and encodes a 563-bp transcript in three exons interrupted by two small introns (44 and 49 bp). The ORF encodes 146 amino acid residues of AVPL pre-propeptide having clear homology and phylogenetic relationship with AVP-related sequences (Fig. 1B). The N-terminal signal peptide is immediately followed by the mature peptide AVPL as CLITNCPRGamide (Fig. 1C). The C-terminus of the putative mature peptide ends with a canonical dibasic cleavage site and an amidation signal (GKR), followed by a moderately conserved, cysteine-rich neurophysin-like sequence (Fig. 1C).

A pure sample of the monomeric peptide F1, CLITNCPRGamide, was available from a batch whose synthesis has been described (Proux et al., 1987). However, for the antiparallel dimer F2 and the parallel dimer D2, only small quantities were available for use as chromatographic standards. We synthesized the dimers non-specifically from F1 using conditions reported to give parallel and antiparallel dimers of the related peptide OT (Yamashiro et al., 1968). Aqueous triethylamine was used to catalyze scrambling of the disulfide bonds, giving a mixture of monomer and dimers. By performing the reaction at ~5-fold higher dilution than in a prior report (Yamashiro et al., 1968), formation of trimers and tetramers was suppressed. For preparative HPLC, 95% ethanol was used as organic modifier, resulting in better separation of the dimers than the conditions shown in Fig. 1A, but poorer separation of the monomer and F2. The sample of antiparallel dimer contained ~1% of the monomer F1 as an impurity.

Four mRNA variants of the *avpl-r* were cloned from larval cDNA in this study. pXOON-AVPLR-8 contains the coding sequence from 24 bp, the site of the second potential translation initiation codon in the ORF, to the stop codon in the pXOON expression vector (Fig. 2A and B). An EST clone, TF1009B5, contains the entire mRNA sequence with a 47 bp predicted intron located between exons 4 and 5, causing a frame shift leading to early termination. This clone was probably an incompletely spliced pre-mRNA, and was modified by deleting the presumed 47 bp intron by using *Pfu* polymerase and phosphorylated primers. The modified clone was named TF1009B5-47. Another EST clone S-3583 contained the entire intact mRNA with an inclusion of 24 bp 5′ of exon 7 (Fig. 2B).

Phylogenetic analysis based on the putative translation of S-3583 (Fig. 2C and D) showed a clear clustering of AVPL-R in the AVP/OT receptor clade, along with receptors for other invertebrate members of this group of neuropeptides, such as annetocin, conopressin, and octopressin. The receptors for gonadotropin releasing hormone (GnRH) and other closely related insect neuropeptide receptors, corazonin receptor, CCAP receptor, and AKH receptors were out-grouped with strong bootstrapping support in the neighbor-joining tree based on distance.

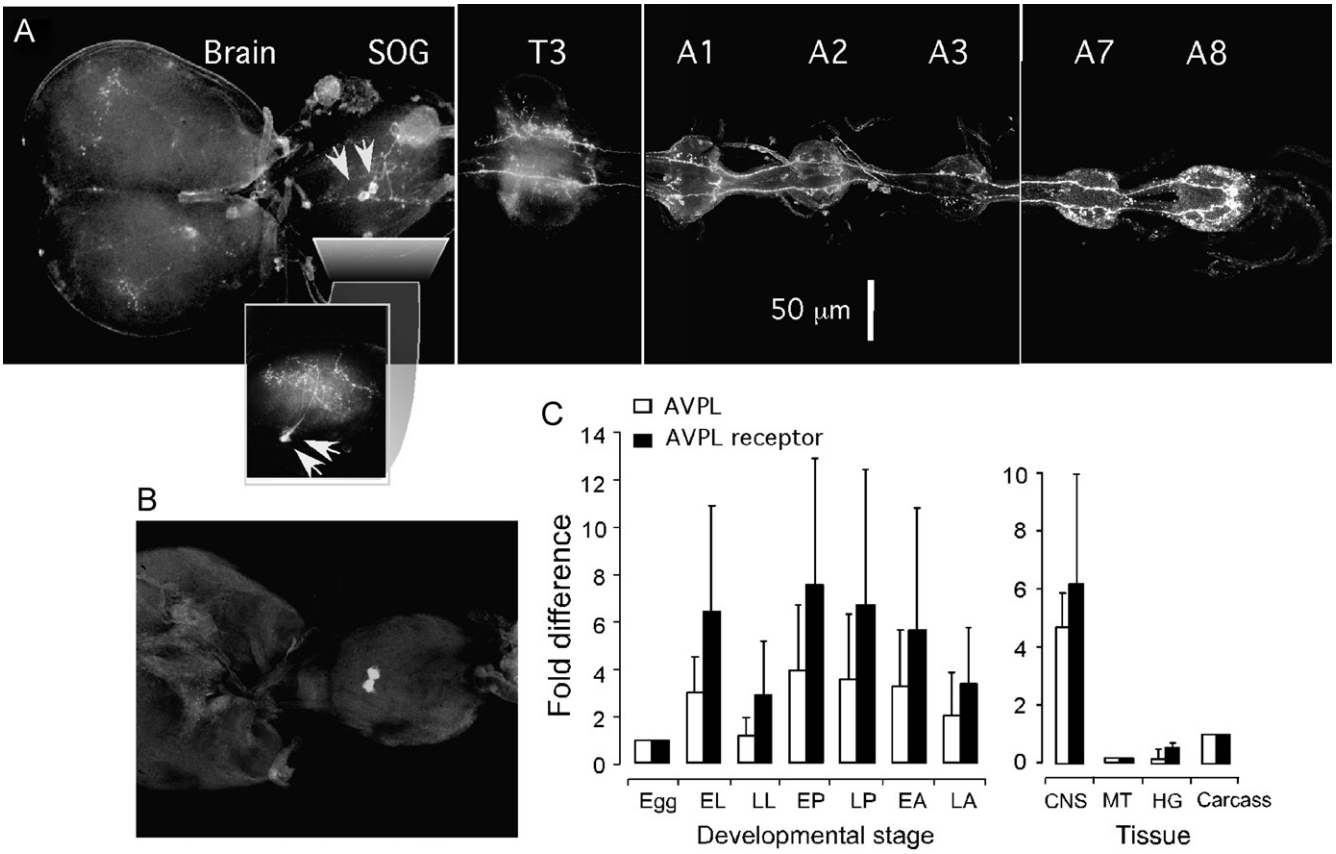

Fig. 3. (A) Immunohistochemistry of *Tribolium* larval brain and subesophageal ganglion for anti-mouse AVP. Arrowheads indicate the anti-AVPL positive cells. (B) *In situ* hybridization using specific *Tribolium avpl* antisense probe. (C) Quantitative reverse transcriptase PCR in different stages and tissues for *avpl* and *avpl-r*. Pools of tissue dissected from three insects were used for total RNA isolation. The averages and standard deviations of three biological replicates are shown. See more details in materials and methods. MT, Malpighian tubules; HG, hindgut.

3.2. Expression patterns of AVPL and its receptor

Immunohistochemistry of AVPL identified a pair of cells on the ventral surface of the subesophageal ganglion (Fig. 3A). The cells have bilaterally symmetric projections directed anteriorly toward the brain and posteriorly toward the terminal abdominal ganglia, each ganglion having extensive varicosities. The immunoreactivity of the pair of subesophageal cells was confirmed by *in situ* hybridization (Fig. 3B), while the sense probe (negative control) did not show any significant staining pattern (data not shown). The immunohistochemical staining patterns and *in situ* hybridization were similar in larvae and adults.

Stage-specific real-time RT-PCR revealed that *avpl* mRNA was continuously detectable from the egg stage, peaking in the EP stage and gradually decreasing in subsequent pupal and adult stages. This pattern is correlated with the pattern for *avpl-r* mRNA. The mRNAs for both genes were detectable mainly in the CNS, while no detectable levels of *avpl-r* expression were found in the MT and hindgut (Fig. 3C).

3.3. Ligand– receptor interactions

Robust luminescent responses were generated on the calcium mobilization assay in heterologously expressed AVPL-R S-3583 clone (Fig. 4A). Among the ligands we tested on the AVPL-R S-3583 clone, AVPL F1 showed the highest activity ($EC_{50} = 1.5$ nM) on the receptors, while F2 and D2 showed lower (7–12 fold and 60–80 fold less, respectively), but still significant levels of activity (Fig. 4B–D). Among the receptor variants tested, TF1009B5, truncated on the 3′ end due to the retention of the 47-bp intron in the pre-mRNA (Fig. 2), was inactive in our assay system. In contrast, TF1009B5-47 and pXOON-AVPR-8 (Fig. 2) showed similar levels of activity to the ligands tested (Fig. 4B and C). The S-3583 clone having the inclusive 24 bp (putative eight amino acid residues) at the 5′ side of exon 7 (Fig. 2) showed the highest sensitivity. Arg-conopressin S (CIIRNCPRGamide, P05487) and OT (CYIQNCPLGamide) had low activity on AVPL receptor variants, whereas mammalian AVP, *Drosophila* AKH, *Tribolium* AKH1 and AKH2 (Li et al., 2008), CCAP, and corazonin were inactive. All the ligands tested in this study on the cell line without *avpl-r* transfection had no activity, indicating there is no endogenous receptor mediating calcium mobilization for those ligands.

3.4. In vivo diuretic activity of AVPL

The *in vivo* effect of AVPL was examined by injection of AVPL into adult *Tribolium*. The immediate excretory response was measured by quantifying the changes in RH in the flow-through air (see material and methods for details). The system was calibrated using known amounts of Ringer's solution. Injections of 50 nl Ringer's solution containing varying doses of AVPL F1 in adult *Tribolium* induced almost immediate diuretic responses of up to ~100 nl of excretion in a dose-dependent manner (Fig. 5A and B). Ten nanomolar *in vivo* concentration (50 nl injection of 100 nM AVPL in 500 nl hemolymph, Fig. 5B) is estimated to be the approximate dose for a minimal effect. Injection of the AVPL variants F2 and D2 at maximum 1 mM concentrations resulted in no detectable responses. Positive controls (8-bromo-cyclic AMP)

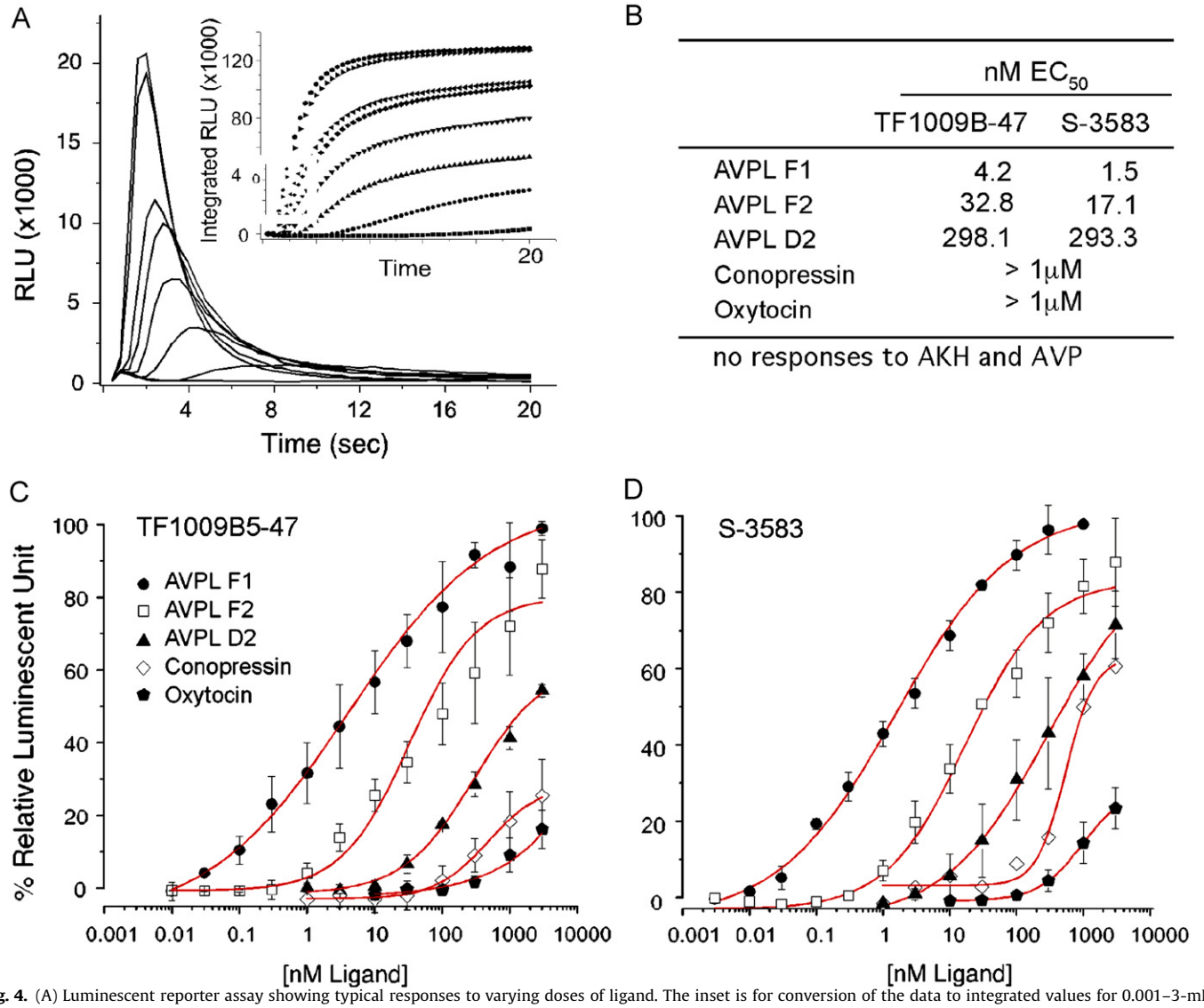

Fig. 4. (A) Luminescent reporter assay showing typical responses to varying doses of ligand. The inset is for conversion of the data to integrated values for 0.001–3-mM ligand concentrations. (B) EC_{50} (nM) of different ligands on two different receptor variants. (C and D) Dose–responses of *Tribolium* AVPL receptor variants to various ligands. Bars indicate standard error for minimum three replicated plates.

and negative controls (Ringer's solution only) produced the expected positive and negative responses. The experiments were highly reproducible in the period from July to October 2006. The reproducibility of the experimental treatments, as well as the positive control (8-Br-cAMP), was reduced after this period for unknown reasons, although similar patterns and statistical significance could be obtained with larger error ranges.

3.5. Secretion assay on isolated MT

We used the MT of another Tenebrionid, *T. molitor*, because unlike *Tribolium*, this insect has large enough MT to perform the secretion assay. Larvae of *T. molitor* also have AVP immunoreactive cells in the subesophageal ganglion (Aikins and Park, unpublished data). The rates of secretion measured over 15 min intervals showed a general pattern of gradual decrease in secretion over time (Fig. 5C). A significant increase in the rate of secretion was induced by the positive control 1 μM of Tenmo-DH_{47}, one of two corticotropin-releasing factor-like diuretic hormones described in *T. molitor* (Furuya et al., 1998). Tenmo-DH_{47} has a sequence identical to that of the *T. castaneum* ortholog, except that the former lacks amidation of the C-terminus. Treatments with another positive control, 8-Br-cAMP, at 1 μM also significantly increased the rate of secretion.

A significant level of increase in the MT secretion rate was observed only in the treatment with the F1 form of AVPL co-incubated with the CNS attached to the corpora cardiaca (CC) and corpora allata (CA). There was no increase in the secretion rate with F2 co-incubated with the CNS-CC/CA, or with F1 alone, or F2 alone. All the ligands were tested at 1 mM (Fig. 5C).

3.6. RNA interference

Reverse transcription-PCR after injection of the LL stage with dsRNA for *avpl* and *avpl-r* indicated efficient suppression of the targeted transcripts in a repeated experiment (Fig. 6). A positive control, RNAi for vermillion causing the loss of eye color, was included to confirm the efficiency of the RNAi. RNAi of *avpl* was also confirmed by the lack of AVPL immunoreactivity in immunohistochemistry with appropriate control samples (two individuals each in two biological replications, data not shown). However, we were unable to detect any defects in development or reproduction during comparisons to controls injected with dsRNA for *vermilion*. Observations and measurements after dsRNA

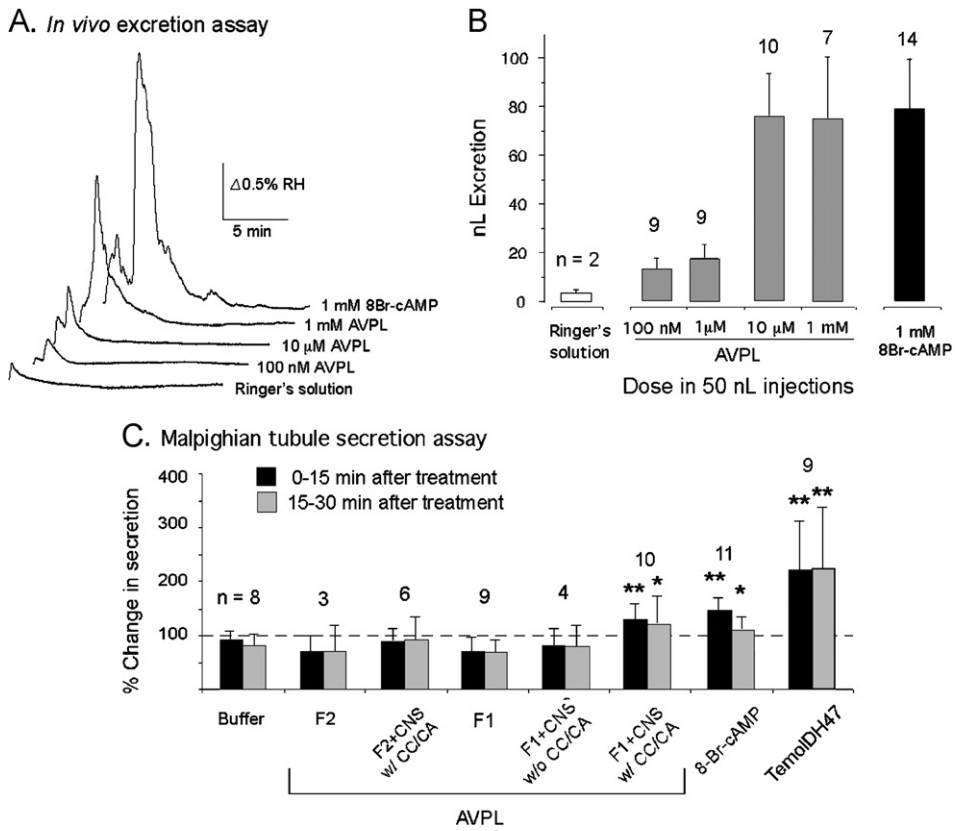

Fig. 5. (A) Examples of *in vivo* excretion induced by injections of AVPL and 8-bromo-cAMP. Injections were made for 50 nL of each indicated concentration. (B) Dose–responses summarized for multiple experiments. (C) Direct assay on *T. molitor* Malpighian tubule secretion. * for $p<0.05$ and ** for $p<0.01$ in the *t*-test assuming equal variance against the value in the negative control (buffer).

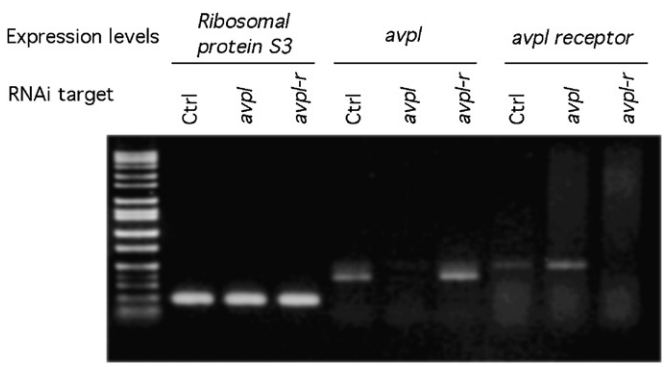

Fig. 6. Reverse transcription PCR showing the suppression of mRNA levels in dsRNA-injected *Tribolium*. Each lane contains the RT-PCR product of two individuals treated with dsRNA after 35 PCR cycles. The picture is representative of two biological replications.

injection included (1) mortality, (2) development time in pupal stage, (3) number and hatch-rate of eggs laid by females after adult development, and (4) mortality in high salt loads (10% NaCl powder mixed in the flour wt/wt). There were no detectable differences in the treated insects by dsRNA compared to the control in those conditions.

4. Discussion

The *Tribolium avpl* gene, the first such gene identified in any insect, encodes a pre-propeptide containing a sequence identical to locust AVPL and a highly conserved neurophysin-like sequence downstream of AVPL, which is known to function for proper processing of AVP in mammals (reviewed in de Bree, 2000). The spatial expression pattern of AVPL was determined by immunohistochemistry and *in situ* hybridization, and revealed a pair of cells in the subesophageal ganglion (Fig. 3), which are apparently homologous to the cells described in the locust (Remy et al., 1979; Tyrer et al., 1993). The *Tribolium* AVPL receptor was grouped in the phylogenetic clade of the AVP receptor GPCRs. Activation of the receptor by AVPL at an EC_{50} of 1.5 nM in the heterologous reporter system suggests that the receptor is indeed the authentic physiological receptor.

4.1. Indirect action of the monomeric AVPL F1 for diuresis

The activities of monomeric (F1), parallel dimeric (F2), and anti-parallel dimeric (D2) AVPL have been controversial. All three forms of AVPL were identified in the locust CNS (Baines et al., 1995). Direct diuretic activity of F2 on the MT was previously reported (Picquot and Proux, 1990; Proux et al., 1987). Another report found inhibitory activity of F1 toward forskolin-stimulated increases in cAMP in isolated neural membranes in locust (Baines et al., 1995). The observation of direct diuretic activity of AVPL variants on the MT in locust was not confirmed by another research group (Coast et al., 1993). The debate on the hormonal diuretic activity of AVPL was further complicated by observations of the pattern of AVPL localization (Tyrer et al., 1993). Bacon's group examined vasopressin-like immunoreactive neurons in 17 Acridoid species and concluded that there is no staining in peripheral fibers (neurohemal organ) except in the subfamily Oedipodinae including *Locusta migratoria*. Tyrer et al. (1993) stated

"…to control water homeostasis cannot be a conserved function… … any conserved role is more likely to be central." Baines et al. (1995) also argued that AVPL is unlikely to have a hormonal function, but may function as a sensory interneuron transmitting signals that indicate light intensity.

In *Tribolium*, we found that AVPL F1 is the most active form on AVPL receptor variants (Fig. 4). F1 was active in the *in vivo* excretion assay and in the secretion assay using the MT of *T. molitor* (Fig. 5), whereas F2 and D2 showed much lower or no activities in all three assay systems. Therefore, we conclude that F1 is the endogenous form of AVPL for receptor activation in *Tribolium*.

We also demonstrated a clear diuretic function of AVPL in the *in vivo* excretion assay in *Tribolium* (Fig. 5); However, we were unable to detect "direct" activity of AVPL F1 on the MT of *T. molitor* in the secretion assay. Interestingly, the preparation incubated with AVPL F1 and the CNS and CC/CA complex with the MT showed a significantly increased rate of secretion (Fig. 5), indicating an indirect action of AVPL F1 through the CNS-CC/CA complex. AVPL F2 with the CNS-CC/CA complex was inactive on the MT.

The dose required for the *in vivo* activity, minimum 10 nM in this study, was higher than previous report in locust, in which 0.36 nM showed activity (Proux et al., 1987). It is possible that the indirect action of AVPL through the nervous system (CNS-CC/CA) may function as a neurotransmitter, for which a generally higher local concentration of ligand is required.

In locust, there have been contradictory results on the diuretic activity of the AVPL. One study showed a strong diuretic activity of AVPL F2 (Proux et al., 1987) while the other showed no activities of both F1 and F2 on the Malpighian tubule (Coast et al., 1993). A possible explanation for the conflicting results could be found in the differences in the experimental preparation: the former used the entire MT including the ampulla while the latter used isolated single Malpighian tubules for the assays. Interestingly, the ampulla was later shown to contain *Locusta* CRF-like diuretic hormone-like immunoreactive endocrine cells and the extract stimulated cAMP elevation in the Malpighian tubule (Montuenga et al., 1996). Therefore, the diuretic activity of AVPL in the locust (Proux et al., 1987) may have been by the indirect action of AVPL to release CRF-like DH from the ampulla endocrine cells, as suggested earlier (Montuenga et al., 1996).

The indirect action hypothesis is further supported by the anti-AVP immunostaining patterns (Fig. 3), limiting the staining within the CNS like the cases shown in a number of locust species (Tyrer et al., 1993). Careful examinations found that the AVPL immunoreactive projections reside within the ganglia and lack detectable nerve projections, leading to a hemoceal releasing site or to peripheral organs in *Tribolium* (i.e., gut and reproductive organs). Real-time RT-PCR found that the expression of *avpl-r* mainly occurs in the CNS ($\sim 10\times$ more than that in gut), though we cannot exclude the possibility that trace levels of receptor expression from other tissue could be important. Summing up the facts, we favor the hypothesis of indirect diuretic activity of AVPL F1, although some of the discrepancies with previous studies could be due to differences in the experimental systems. In *Tribolium*, we propose that AVPL stimulates (directly or indirectly) the secretion of diffusible diuretic factors from the CNS or CC/CA, which act on the MT for diuresis. In particular, the CC of *T. molitor* is known to contain Tenmo-DH_{37} (Wiehart et al., 2002), which is a potent diuretic factor acting through cAMP elevation in the MT (Furuya et al., 1995). Further investigations will be required to completely understand the mechanism of AVPL action.

4.2. Evolution of AVPL and AVPL-R

Neuropeptide genes often rapidly evolve, leaving only a short sequence motif useful for phylogenetic inference. For this reason, the sequences for the cognate receptors, which contain more informative sequences, are often used for deducing the evolutionary history of a particular signaling system. Likewise, the phylogeny of the receptor for AVPL revealed that the insect receptors for crustacean cardioactive peptide (CCAP), adipokinetic hormone (AKH), corazonin, and vertebrate GnRH are ancestrally related to the AVP/OT receptors (Park et al., 2002).

As the coevolution between ligands and receptors predicts, the evolutionary relationship of ligands in the AVP/OT group is generally mirrored in the phylogeny of cognate receptors (Figs. 1 and 2) with minor exceptions, particularly in the vertebrates. Indeed, the evolution of OT, isotocin, vasotocin, and AVP and their cognate receptors are thought to be complex, including the possibility of concerted evolution via recombination between the closely located genes *oxytocin* and *vasopressin* (Mohr et al., 1995).

As was mentioned earlier, AVPL and its receptor were found only in the *Tribolium* genome, and are absent in other insect genome sequences including *Drosophila*, *Anopheles*, *Apis*, and *Bombyx*, which all happen to be holometabolous insects. Another exception is the recently sequenced holometabolous species *Nasonia,* whose genome reveals putative *avpl* and *avpl-r* genes. The presence of vasopressin-immunoreactive neurons was reported for locusts, cockroaches, mantids, and the Colorado potato beetle (Davis and Hildebrand, 1992; Veenstra, 1984), whereas such activity is absent in *B. mori* (Remy et al., 1979). The lack of sequences encoding both the ligand and the receptor in multiple species and the general consensus regarding the presence/lack of immunoreactive cells strongly support the conclusion that the gene is truly missing in most holometabolous insects examined with the exception of Coleoptera and of *Nasonia*. Recent studies on the phylogeny of insects using multiple genes concluded that the Order Hymenoptera is indeed the most basal lineage of holometabolous insect (Savard et al., 2006), indicating that the loss of AVPL and its receptor in holometabolous insects may have happened at least twice, assuming that the absence of the genes in the honeybee is true.

The loss of the AVPL signaling system in other holometabolous insects but its presence in *Tribolium* (Coleoptera) and *Nasonia* (Hymenoptera) is surprising, considering that AVP/OT is highly conserved across the bilaterians in the Metazoa. In *Tribolium*, RNAi of *avpl* or *avpl-r* did not show any noticeable abnormal phenotype in our assay system. Taken together, a favored explanation is that the AVPL signaling system had been near dispensable in the ancestors of holometabolous insects, and eventually was lost in the majority of holometabolous lineages, while the AVPL functions, including indirect stimulation of diuresis, are still retained in *Tribolium* and *Locusta*.

We recently learned that another group has published a paper describing the identification of AVPL and its receptor, which is a part of the finding included in this manuscript (Stafflinger et al., 2008). Although this paper did not test either dimeric ligand, or contain any information on the biological function of AVPL, the paper supports many of our findings described in this manuscript.

Acknowledgments

We thank Ms. Sherin Pattarettu for HPLC separation of products of the non-optimized synthesis. This study was funded by USDA-NRI-CRSEES 2007-35604-17759 and the Nevada Agriculture Experiment Station. This is Contribution

Number 08-215-A from the Kansas Agricultural Experimental Station. The sequences for *avpl* and *avpl receptor* were deposited in GenBank with accession numbers EF222295 and EF222296, respectively. All programs and services of the US Department of Agriculture are offered on a nondiscriminatory basis, without regard to race, color, national origin, religion, sex, age, marital status, or handicap.

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