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Molecular basis of the dopaminergic system in the cricket *Gryllus bimaculatus*

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Running title: dopamine related genes in the cricket

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ABSTRACT

In insects, dopamine modulates various aspects of behavior such as learning and memory, arousal and locomotion, and is also a precursor of melanin. To elucidate the molecular basis of the dopaminergic system in the field cricket *Gryllus bimaculatus* DeGeer, we identified genes involved in dopamine biosynthesis, signal transduction and dopamine re-uptake in the cricket. Complementary DNA of two isoforms of tyrosine hydroxylase (TH), which convert tyrosine into L-3,4-dihydroxyphenylalanine (L-DOPA), were isolated from the cricket brain cDNA library. In addition, four dopamine receptor genes (*Dop1*, *Dop2*, *Dop3* and *DopEcR*) and a high-affinity dopamine transporter (*DAT*) gene were identified. The two TH isoforms contained isoform-specific regions in the regulatory ACT domain, and showed differential expression patterns in different tissues. In addition, the dopamine receptor genes had a receptor subtype-specific distribution: the *Dop1*, *Dop2* and *DopEcR* genes were broadly expressed in various tissues at differential expression levels, and the *Dop3* gene was restrictedly expressed in neuronal tissues and the testicles. Our findings provide a fundamental basis for understanding the dopaminergic regulation of diverse physiological processes in the cricket.

Keywords: dopamine, tyrosine hydroxylase, dopamine receptors, high-affinity dopamine transporter, *Gryllus bimaculatus*

1 INTRODUCTION

2 Biogenic amines function as intracellular messenger molecules that play essential
3 roles in regulating physiological processes and in controlling various behaviors in insects
4 (Evans, 1980; Roeder, 2005). Pharmacological and behavioral studies have been carried out
5 to investigate roles of the biogenic amine system in several insects including the field cricket
6 *Gryllus bimaculatus* DeGeer. For example, pharmacological manipulation of the
7 octopaminergic and dopaminergic systems revealed that these two biogenic amines are
8 involved in appetitive and aversive learning in the cricket, respectively (Unoki et al., 2005;
9 Unoki et al., 2006; Mizunami et al., 2009). Application of octopamine receptor antagonists
10 and dopamine/octopamine depletion induced by blocking their biosynthesis with α -methyl-p-
11 tyrosine lead to a decrease in aggression in male crickets (Stevenson et al., 2005; Rillich and
12 Stevenson, 2011; Rillich et al., 2011). Recently, transgenic techniques became applicable in
13 *G. bimaculatus* (Nakamura et al., 2008), which allow that physiological roles of the biogenic
14 amine system in the cricket nervous system can be analyzed using a neurogenetic approach.
15 However, although physiological and pharmacological studies have been extensively carried
16 out to elucidate the functional roles of the cricket biogenic amine system, little is known
17 about its molecular basis. In a previous report, we identified genes involved in the cricket
18 serotonergic system and examined their expression patterns (Watanabe et al., 2011). Here, we
19 focus on the dopaminergic system and investigate the molecular basis of the dopaminergic
20 system in *G. bimaculatus*.

21 In insects, dopamine functions as a neurotransmitter/neurohormone that is
22 synthesized in and released from dopaminergic neurons in the nervous system, and also
23 serves as a precursor of melanin that plays important roles in cuticular
24 sclerotization/pigmentation and in the innate immune system. Dopamine biosynthesis
25 requires two enzymes, tyrosine hydroxylase (TH) and aromatic L-amino acid decarboxylase

(AADC, also known as dopa decarboxylase (DDC)). As the initial and rate-limiting step of dopamine biosynthesis, TH converts L-tyrosine into L-3,4-dihydroxyphenylalanine (L-DOPA). Then, AADC converts L-DOPA into dopamine. AADC is involved not only in dopamine biosynthesis but also in serotonin biosynthesis, and is expressed ubiquitously in cricket tissues (Watanabe et al., 2011). Once dopamine is released extracellularly as a neurotransmitter or neurohormone, it is received by dopamine receptors on the plasma membrane of target cells, or undergoes re-uptake via the high-affinity dopamine transporter. Insect dopamine receptors belong to the seven-transmembrane segments (7TM)-containing G protein-coupled receptor (GPCR) superfamily, and are classified into four subtypes: the D₁-like dopamine receptors (Dop1), invertebrate dopamine receptors (INDRs, also known as Dop2), the D₂-like dopamine receptors (Dop3) and the dopamine/ecdysteroid receptors (DopEcR) (Mustard et al., 2005; Srivastava et al., 2005). Once dopamine receptors are activated by dopamine, downstream signaling systems, such as the cyclic adenosine monophosphate (cAMP) second messenger pathway and the inositol trisphosphate (IP₃) second messenger pathway, are activated. The high-affinity dopamine transporter (DAT) is a 12-TM spanning Na⁺/Cl⁻-dependent sodium: neurotransmitter symporter that belongs to the solute carrier family of membrane transport proteins.

In the present study, we identified six genes involved in biosynthesis, transduction and transport of dopamine expressed in the brain of the field cricket *G. bimaculatus*. We obtained a partial open reading frame (ORF) clone of two isoforms of the TH gene, full-length ORF clones of three dopamine receptor genes (*Dop1*, *Dop2* and *DopEcR*), a partial ORF clone of a D₂-like dopamine receptor gene (*Dop3*) and a full-length ORF clone of a *Gryllus* DAT gene. Expression analysis of the genes revealed an isoform-specific distribution of the two TH isoforms and a receptor-subtype specific distribution of the dopamine receptor genes. Our data demonstrate that the dopaminergic system is

1 widespread in the CNS and in peripheral tissues of the cricket.

1 MATERIALS AND METHODS

2 Animals

3 Crickets *Gryllus bimaculatus* DeGeer were reared on a 14-h:10-h light/dark cycle at
4 27°C. They were fed a diet of insect food pellet (Oriental Yeast Co., Tokyo, Japan), chopped
5 carrot and water *ad libitum*.

7 Complementary DNA Cloning of *TH*, *Dop1*, *Dop2*, *Dop3*, *DopEcR* and *DAT* genes

8 First, we cloned partial cDNAs of target genes by reverse transcriptase-polymerase
9 chain reaction (RT-PCR). Complementary DNA synthesis was carried out according to the
10 experimental procedure described previously (Watanabe et al., 2011). In order to design gene
11 specific primers to clone a partial cDNA of the *Gryllus TH* gene, we searched EST
12 (expressed sequence tag) clones corresponding to *Gryllus TH* on the GenBank database, and
13 obtained an EST sequence encoding the N-terminus of TH protein (GenBank accession
14 number: AK278042). To obtain a cDNA fragment encoding the C-terminal region of TH
15 protein, we designed a forward gene specific primer (GSP) and a reverse degenerate primer
16 on the basis of the nucleotide sequence of the *Gryllus TH* EST clone and a conserved amino
17 acid sequence (MSRPFEV) among the insect TH proteins, respectively. The partial cDNA
18 fragments of *Gryllus Dop1* and *Dop2* genes were cloned by Hamada et al (2009). A partial
19 cDNA fragment of the *Gryllus Dop3* gene was amplified as a side product in our previous
20 study (Watanabe et al., 2011). Partial cDNA fragments of the *Gryllus DopEcR* and *DAT*
21 genes were amplified using degenerate primers designed on the basis of conserved amino
22 acid sequences among the insect DopEcR proteins (SVYTFMWI and CQCWMV) and DAT
23 proteins (GIPLFYM and YVDFYYNVII), respectively.

24 Next, to obtain the 5' and 3' region of target mRNAs, we performed 5' and 3' rapid
25 amplification of cDNA ends (RACE) using the FirstChoice RLM-RACE kit (Ambion,

Austin, TX, USA). Finally, we performed RT-PCR to amplify the cDNA fragments containing the full-length or partial open reading frame (ORF) of the genes. We determined nucleotide sequences of at least three independent cDNA clones, and registered their consensus sequences to GenBank. All PCRs were carried out according to the experimental procedure described previously (Watanabe et al., 2011). The primers used to amplify the cDNAs containing the full-length or partial ORF are listed in Table 1.

Sequence comparison, prediction of transmembrane segments, PEST domain, N-glycosylation sites and phosphorylation sites

The deduced full-length amino acid sequences of *Gryllus* Dop1, Dop2, DopEcR and DAT, and the deduced partial amino acid sequences of *Gryllus* TH and Dop3 were compared with those of the corresponding parts of the known homologous genes of other species by using the MAFFT or MUSCLE algorithms on the Geneious 5.6 program (Drummond et al., 2011). The GenBank accession numbers of the proteins used for the comparison are listed in Table 2. The transmembrane regions of the dopamine receptors and of DAT were predicted by the TMHMM v. 2.0 program (Sonnhammer et al., 1998; <http://www.cbs.dtu.dk/services/TMHMM/>). The PEST domain was searched in insect TH proteins using a web-based algorithm, PESTFind (<http://emboss.bioinformatics.nl/cgi-bin/emboss/pepfind>). Potential N-glycosylation and phosphorylation sites were predicted using the NetNGlyc 1.0 program (<http://www.cbs.dtu.dk/services/NetNGlyc>) and the NetPhos 2.0 program (Blom et al., 1999; <http://www.cbs.dtu.dk/services/NetPhos/>), respectively.

Molecular phylogenetic analyses

Molecular phylogenetic trees of the *Gryllus TH*, *Dop1*, *Dop2*, *DopEcR* and *DAT* genes were calculated using their deduced amino acid sequences. Because the deduced amino acid sequence of the partial cDNA of *Gryllus Dop3* is too short to construct phylogenetic tree, we constructed the molecular phylogenetic tree of the *Gryllus* D₂-like dopamine receptor (*Dop3*) gene using their nucleotide sequences corresponding to the protein-coding region. The protein sequences were aligned with the corresponding parts of the known homologous genes of other species by using the MAFFT or MUSCLE algorithm on the Geneious 5.6 program (Drummond et al., 2011). The cDNA sequence of *Gryllus Dop3* encoding the C-terminus of the protein was aligned with the corresponding parts of the known homologous genes of other species by using the ClustalW algorithm on the Geneious 5.6 program. Phylogenetic trees were constructed from the aligned sequences by the bootstrap neighbor-joining algorithms on the MEGA 5 program (Tamura et al., 2011) and visualized with the Geneious 5.5 program. The GenBank accession numbers of the proteins/nucleotides used for the comparison are listed in Table 2.

Tissue-specific expression analysis of *TH*, *Dop1*, *Dop2*, *Dop3*, *DopEcR* and *DAT* genes

RT-PCR analysis was performed to assess the tissue-specific expression of the *Gryllus TH*, *Dop1*, *Dop2*, *Dop3*, *DopEcR* and *DAT* genes. The following tissues were subjected to expression analysis: (1) the central brain, (2) suboesophageal ganglion (SOG), (3) optic lobe (lamina + medulla) and retina, (4) corpus cardiacum-corpora allata complex (CC + CA), (5) thoracic muscles, (6) salivary glands, (7) midgut, (8) Malpighian tubules, (9) testes and (10) ovaries. RNA extraction and reverse transcription were carried out according to the experimental procedure described previously (Watanabe et al., 2011). Briefly, tissue samples were dissected from 1-week-old adult crickets, and total RNA was extracted with TRIzol reagent (Invitrogen). After DNase treatment, 1 µg of each total RNA was reverse-

transcribed in a 20 µl reaction using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Tokyo, Japan). A random hexamer and an anchored oligo(dT)₁₈ primer were used as primers. PCR was carried out using *Ex taq* polymerase (TaKaRa, Shiga, Japan). 0.2 µl of cDNA solution was added to a 10 µl PCR reaction. PCR amplification of target genes was performed for 35 cycles at 96°C for 15 s, 58 °C for 15 s, and 72°C for 60 s, followed by a final extension at 72°C for 5 min. PCR products were run through a 1.5% agarose gel and visualized by etidium brimide. The *Ef1alpha* gene, which is ubiquitously and stably expressed in all examined tissue (Watanabe et al., 2011), was amplified as an internal control gene. To confirm that genomic DNA was not present in cDNA solutions, we amplified the *Ef1alpha* gene and checked the absence of an amplification product in the RT negative controls (RT(-)). Primers used for the RT-PCR analysis are listed in Table 1.

RESULTS

Identification and expression analyses of *Gryllus* tyrosine hydroxylase gene

Nucleotide sequence and structural features of *Gryllus* TH isoforms: We obtained partial cDNA fragments of two isoforms of tyrosine hydroxylase gene (*TH*) expressed in the cricket. We performed RT-PCR with the gene-specific primers (GSPs) and obtained a 1408-bp cDNA fragment of the *TH-A* isoform and a 1606-bp cDNA fragment of the *TH-B* isoform (GenBank accession numbers: AB720738 and AB720737, respectively). These genes have the following properties: *TH-A* isoform, partial ORF = 29–1408 bp, protein product = 460 amino acids; *TH-B* isoform, partial ORF = 29–1606 bp, protein product = 526 amino acids (Figure 1A and B). Several functional domains and residues, such as the ACT domain, catalytic domain and several catalytic residues are conserved in both *Gryllus* TH proteins (Figure 1A). In addition, two conserved arginine residues (Arg²⁹ and Arg³⁰), important for catecholamine-mediated inhibition of enzyme activity (Nakashima et al., 1999; Nakashima et al., 2000), were conserved in the N-terminal region of both *Gryllus* TH proteins (Figure 1A). We failed to obtain a cDNA clone of the 3' region of the *TH* ORF corresponding to the C-terminal coiled-coil domain important for dimerization. The *Gryllus* TH-B isoform contains a 66-amino-acid insertion in the ACT domain. The position of this insertion was conserved in other insect TH-B isoforms such as those found in *Mythimna separata* and *Drosophila melanogaster* (Birman et al., 1994; Ninomiya and Hayakawa, 2007) (Figure 1B). The inserted sequences of the three insect TH-B isoforms are predicted as the PEST domain that is found in many rapidly degraded proteins (Rogers et al., 1986).

A comparison of the deduced amino acid sequences of the *Gryllus* TH isoforms with those of other insect amino acid hydroxylases indicates that the *Gryllus* TH isoforms are closely related to other known insect THs (*Gryllus* TH-A is 73.1% identical to *Drosophila* pale-A (NP_476897), 74.1% identical to *Mythimna* TH (type 2, brain form; BAF32574) and

75.1% identical to *Apis* TH (NP_001011633)). The molecular phylogenetic analysis of aromatic amino acid hydroxylase genes also indicates that *Gryllus* TH is closely related to the insect TH protein (Figure 2).

Tissue-specific expression of *Gryllus* TH isoforms: We examined the tissue-specific expression pattern of total TH transcript and each TH isoform in the cricket by using RT-PCR (Figure 3). Our results indicate an isoform-specific expression pattern of the *Gryllus* TH gene: the *Gryllus* TH-A isoform is predominantly expressed in neural tissues, whereas the *Gryllus* TH-B isoform is ubiquitously expressed in all examined tissues. An intensely stained band of PCR product of the *Gryllus* TH common region (cDNA fragments corresponding to the catalytic domain) was detected in the lanes of the central brain, SOG and the ovaries. A weaker stained band of PCR product of the *Gryllus* TH common region was detected in the lanes of the optic lobe + retina, CC + CA, thoracic muscles, salivary gland and testes. An intensely stained band of PCR product of the *Gryllus* TH-A isoform was detected in the lanes of the central brain, SOG, optic lobe + retina and CC + CA. A weaker stained band of PCR product of the *Gryllus* TH-A isoform was detected in the lanes of the thoracic muscles and testicle. An intensely stained band of PCR product of the *Gryllus* TH-B isoform was detected in the lanes of the thoracic muscles, testicle and ovary. A weaker stained band of PCR product of the *Gryllus* TH-B isoform was detected in the lanes of the other tissues except for the midgut and Malpighian tubulus.

Identification and expression analysis of three *Gryllus* dopamine receptor genes

D₁-like and invertebrate dopamine receptors: We identified the D₁-like dopamine receptor gene (*Dop1*) and the invertebrate dopamine receptor gene (*Dop2*) in the cricket. We performed RT-PCR with the GSPs designed at the 5' and 3' UTRs of the genes, and obtained a 1478-bp cDNA fragment of *Gryllus Dop1*, and a 1458-bp cDNA fragment of *Gryllus Dop2*

(GenBank accession numbers: AB720739 and AB720740, respectively). These genes have the following properties: *Dop1*, full-length ORF = 9–1463 bp, protein product = 485 amino acids; *Dop2*, full-length ORF = 50–1447 bp, protein product = 465 amino acids (Figure 4A and B).

The cDNA fragment of *Gryllus Dop1* encoded the seven transmembrane (TM) segments (TM1–TM7 in Figure 4A). The N-terminal extracellular region contained a putative N-glycosylation site. The first, second, and third cytoplasmic loops (the region between TM1-TM2, TM3-TM4 and TM5-TM6, respectively) and the C-terminal intracellular region contained several potential phosphorylation sites. The second cytoplasmic loop contained a conserved DRY motif. The cDNA fragment of *Gryllus Dop2* encoded the seven transmembrane (TM) segments (TM1-TM7 in Figure 4B). The N-terminal extracellular region contained three putative N-glycosylation sites. The first and third cytoplasmic loops (the region between TM1-TM2 and TM5-TM6, respectively) and the C-terminal intracellular region contained several potential phosphorylation sites. The second cytoplasmic loop contained a conserved DRY motif.

A comparison of the deduced amino acid sequences of *Gryllus Dop1* and *Dop2* with those of other insect G-protein coupled receptors indicates that *Gryllus Dop1* is closely related to other known insect Dop1 (53.2% identical to *Bombyx Dop1* (NP_001108459), 53.2% identical to *Apis Dop1* (NP_001011595) and 53.0% identical to *Drosophila Dop1* (NP_477007)), and that *Gryllus Dop2* is closely related to other known insect Dop2 (68.7% identical to *Bombyx Dop2* (NP_001108338), 66.4% identical to *Apis Dop2* (NP_001011567) and 61.2% identical to *Drosophila Dop2* (NP_733299)). The molecular phylogenetic analysis of the D₁-like dopamine receptors and the INDRs also indicates that *Gryllus Dop1* and *Dop2* are closely related to insect Dop1 and Dop2 proteins, respectively (Figure 5).

D₂-like dopamine receptor: We identified an insect D₂-like dopamine receptor gene, *Dop3*, expressed in the cricket brain. We performed RT-PCR with the GSPs and obtained a 484-bp cDNA fragment of the *Gryllus Dop3* gene (GenBank accession number: AB720741). This clone contains an ORF spanning 2–201 bp, resulting in a protein product of 63 amino acids. The cDNA fragment of *Gryllus Dop3* encoded the two transmembrane (TM) segments (TM6 and TM7 in Figure 6A). In addition, we isolated another cDNA clone that differed by an alternatively spliced intron within the 3' UTR (*Gryllus Dop3* long 3' UTR variant; GenBank accession number: AB720740).

Comparison of the deduced amino acid sequence of *Gryllus Dop3* with those of the corresponding part of other insect G-protein coupled receptors indicates that *Gryllus Dop3* is closely related to other known insect D₂-like dopamine receptors (78.5% identical to *Apis Dop3* (NP_001014983), 85.1% identical to *Tribolium Dop3* (XP_969037), and 74.3% identical to *Drosophila DDR2* (NP_001014760)). The molecular phylogenetic analysis of the D₂-like dopamine receptors also indicates that *Gryllus Dop3* is closely related to insect D₂-like receptors (Figure 6B).

Dopamine/ecdysteroid receptor: We identified an insect dopamine/ecdysteroid receptor gene, *DopEcR*, in the cricket. We performed RT-PCR with the GSPs designed on the 5' and 3' UTRs of the gene, and obtained a 1046-bp cDNA fragment of *Gryllus DopEcR* (GenBank accession number: AB720743). This clone contains an ORF spanning 23–1003 bp, resulting in a protein product of 326 amino acids (Figure 7A). The cDNA fragment of *Gryllus DopEcR* encoded the seven transmembrane (TM) segments (TM1–TM7 in Figure 7A). The second cytoplasmic loop contained a DRY motif.

Comparison of the deduced amino acid sequences of *Gryllus DopEcR* with those of other insect G-protein coupled receptors indicates that the *Gryllus DopEcR* is closely related to other known insect DopEcR (72.4% identical to *Apis DopEcR* (XP_396491), 66.3%

identical to *Anopheles* DopEcR (XP_315694) and 66.9% identical to *Drosophila* DopEcR (NP_647897). The molecular phylogenetic analysis of the DopEcR indicates that the *Gryllus* DopEcR is closely related to other insect DopEcR proteins (Figure 7B).

Tissue-specific expression of four dopamine receptor genes: We examined the tissue-specific expression pattern of the four identified dopamine receptor genes by using RT-PCR. Our results indicate that the *Gryllus* dopamine receptor genes exhibit receptor subtype-specific distributions (Figure 8): *Gryllus Dop1* and *Dop2* genes were ubiquitously expressed in all examined tissues, whereas the *Gryllus Dop3* and *DopEcR* genes showed restricted expression in specific tissues. An intensely stained band of PCR product of *Gryllus Dop1* gene was detected in the lanes of the central brain, SOG, CC + CA, salivary gland, midgut, testicle and ovaries. A weaker stained band of PCR product of *Gryllus Dop1* gene was detected in the lanes of the optic lobe + retina, thoracic muscles and Malpighian tubules. An intensely stained band of PCR product of *Gryllus Dop2* gene was detected in the lanes of the central brain, SOG, CC + CA, thoracic muscles, midgut, testicle and ovaries. A weaker stained band of PCR product of *Gryllus Dop2* gene was detected in the lanes of the optic lobe + retina, salivary gland and Malpighian tubules. An intensely stained band of PCR product of *Gryllus Dop3* gene was detected in the lanes of the central brain, SOG and testicle. An intensely stained band of PCR product of the *Gryllus DopEcR* gene was detected in the lanes of the central brain, SOG, CC + CA, thoracic muscles, midgut, testicle and ovaries. A weaker stained band of PCR product of *Gryllus DopEcR* gene was detected in the lanes of the salivary gland. Expression on *Gryllus DopEcR* gene was not detected in the lane of the Malpighian tubules.

Identification and expression analysis of a *Gryllus* high-affinity dopamine transporter gene

We identified a high-affinity dopamine transporter gene, *DAT*, expressed in the cricket brain. We performed RT-PCR with the GSPs designed on the 5' and 3' UTRs of the *Gryllus DAT* gene and obtained an 1880-bp cDNA fragment (GenBank accession number: AB720744). This clone contains an ORF spanning 17–1855 bp, resulting in a protein product of 612 amino acids. The cDNA fragment of *Gryllus DAT* encoded the twelve TMs (TM1–TM12 in Figure 9A). The second and third extracellular loop (the region between TM3-TM4 and TM5-TM6) contained potential N-glycosylation sites. The N-terminal intracellular region and the second intracellular loop (the region between TM4-TM5) contained potential phosphorylation sites. In addition, two cysteine residues that are required for *DAT* biosynthesis and/or its delivery to the cell surface (Chen et al., 2007) were conserved in the second extracellular loop (“S-S bond” in Figure 9A).

A comparison of the deduced amino acid sequence of *Gryllus DAT* with those of other insect monoamine transporters indicates that *Gryllus DAT* is closely related to other known insect *DAT* (75.7% identical to *Apis DAT* (NP_001139210), 74.3% identical to *Bombyx DAT* (NP_001037362) and 72.0% identical to *Drosophila DAT* (NP_523763)). The molecular phylogenetic analysis of biogenic amine transporter proteins, including the serotonin transporter (SERT), octopamine transporter (OAT) and noradrenaline transporter (NAT), also indicates that the *Gryllus DAT* is closely related to the insect *DAT* proteins (Figure 9B).

Next, we performed RT-PCR analyses to investigate the tissue-specific expression of the *Gryllus DAT* gene. The PCR product of *Gryllus DAT* was detected in the lanes of all examined tissues (Figure 10).

DISCUSSION

Multiple TH protein isoforms were found in vertebrates (four isoforms in human, two isoforms in monkey) (Nagatsu, 1989; Lewis et al., 1993; Lewis et al., 1994; Haycock, 2002), which are produced by alternative splicing at the N-terminal portion of the regulatory ACT domain. In the present study, we isolated partial cDNA fragments of two *TH* isoforms (the short variant, *TH-A*; the long variant, *TH-B*) generated by alternative splicing in the middle of the ACT domain. Similar alternatively spliced forms of the *TH* gene were identified in *D. melanogaster* and the armyworm *M. separata* (Birman et al., 1994; Ninomiya and Hayakawa, 2007). In the cricket, the *TH-A* isoform is predominantly expressed in the CNS, whereas the *TH-B* isoform is ubiquitously expressed. In *D. melanogaster* and *M. separata*, the corresponding *TH* isoforms show similar expression patterns (Friggi-Grelín et al., 2003; Ninomiya and Hayakawa, 2007). These data suggest that the existence of two TH isoforms and the isoform-specific expression pattern of the *TH* gene are evolutionary conserved in insects. Evolutionary conserved tissue-specific distribution of two *TH* isoforms suggests that the *TH-A* isoform is predominantly responsible for synthesis of the neurotransmitter dopamine, while the *TH-B* isoform is also responsible for synthesis of dopamine as a precursor of melanin, which is required for cuticular tanning/sclerotization and the innate immune system.

The inserted sequence of the *Gryllus* *TH-B* isoform and its homologous sequences in *D. melanogaster* and *M. Separata* are predicted as the PEST domain (Figure 1B). The PEST domain is found in many rapidly degraded proteins (Rogers et al., 1986). The degradation of a PEST-containing protein is mediated via the ubiquitin/proteasome system or calpain (Shumway et al., 1999; Reverte et al., 2001; Spencer et al., 2004). Moreover, recombinant *Drosophila* *TH* isoforms exhibited distinct enzymatic properties *in vitro* (i.e. cofactor

binding) (Vié et al., 1999). Further biochemical studies are necessary to reveal the functional roles of the inserted region of the insect TH-B isoforms.

The change of the dopamine level in the nervous systems has been reported in several insects. In the brain of the wood ant *Formica japonica*, dopamine levels decrease in response to starvation stress and aging (Wada-Katsumata et al., 2011; Aonuma and Watanabe, 2012). In the honeybee, dopamine level in the optic lobes changes with the light-dark cycle (Carrington et al., 2007). **In social insects, changes in brain dopamine levels are associated with division of labor, reproductive status and caste (Brandes et al., 1990; Taylor et al., 1992; Schulz and Robinson, 1999; Bloch et al., 2000; Harano et al., 2005).** Since TH catalyzes the rate-limiting step of dopamine biosynthesis, regulation of the TH enzymatic activity is one of the key mechanisms underlying the change of the dopamine level in the CNS. The N-terminal region of the TH protein is important for the regulation of its enzyme activity (Nakashima et al., 2009; Nakashima et al., 1999; Nakashima et al., 2000) and protein stability (Nakashima et al., 2011). The N-terminal region of the *Gryllus* TH isoforms contains several putative phosphorylation sites (Ser³², Ser⁵⁰, Tyr⁶⁵ and Ser⁷⁸) which are conserved in *Drosophila* TH (pale, see Figure 1). The Ser³² is conserved in TH proteins of both vertebrates and invertebrates, and enzymatic activity of *Drosophila* TH is increased by phosphorylation at Ser³² *in vitro* (Vié et al., 1999). The Ser⁴⁰ residue of vertebrate TH proteins, which is homologous to the Ser³² of insect TH, is phosphorylated by various kinases including PKA, PKG and PKC (Dunkley et al., 2004). Further studies will reveal the cellular mechanisms underlying the changes in dopamine levels in the insect CNS.

Except for a dopamine-gated chloride channel found in nematodes [LGC-53 in *Caenorhabditis elegans* (Ringstad et al., 2009) and HcGGR3 in *Haemonchus contortus* (Vijayaraghava et al., 2009)], all dopamine receptors belong to the GPCR superfamily, and are classified into three subtypes: the D₁-like receptors, INDRs and D₂-like receptors.

1 Recently another GPCR-type receptor, DopEcR, was identified in *D. melanogaster*, which
2 shows high affinity for dopamine as well as ecdysteroids (Srivastava et al., 2005). D₁-like
3 receptors are coupled to G_{as} protein, which subsequently activates adenylyl cyclase to
4 produce cAMP, and the INDRs are coupled to Ca²⁺ signaling as well as cAMP (Beggs et al.,
5 2011). On the other hand, D₂-like dopamine receptors are coupled to G_{ai} protein that inhibits
6 adenylyl cyclase. D₂-like dopamine receptors can also activate the mitogen-activated protein
7 kinase (MAPK) pathway (Yan et al., 1999). Activation of DopEcR by dopamine leads to an
8 increase in intracellular cAMP levels as well as activation of the phosphoinositol 3-phosphate
9 pathway. Ecdysteroids, in contrast, inhibit the effects of dopamine and induce the activation
10 of the MAPK pathway (Srivastava et al., 2005). In the present study, we searched GPCRs
11 structurally associated with *Gryllus* DopEcR in order to construct a molecular phylogenetic
12 tree of DopEcR, and found that the vertebrate GPR21 and GPR52 genes are most similar to
13 insect DopEcR. GPR21 and GPR52 were first identified from the human expressed sequence
14 tags database and genome database (O'Dowd et al., 1997; Sawzdargo et al., 1999), and their
15 homologues were then found in other vertebrate genomes (see Table 2). Although the natural
16 agonists, downstream signal transduction pathways and physiological roles of the vertebrate
17 GPR21 and GPR52 are still unknown, the mRNA of human GPR21 is expressed in several
18 brain areas (e.g. the frontal cortex, caudate nucleus, putamen, thalamus) (O'Dowd et al.,
19 1997). Gene function analyses of the DopEcR/GPR21/GPR52 family are necessary to reveal
20 the physiological roles of the novel GPCR family.

21 Dopamine is widely distributed in the CNS and in peripheral tissues of both
22 vertebrates and invertebrates. Application of agonists/antagonists of specific dopamine
23 receptors has revealed physiological functions of dopamine in various insect tissues. To
24 elucidate the sites of dopamine action in the cricket, we investigated the tissue-specific
25 expression of four dopamine receptor genes in the tissue of adult crickets.

1 **Central nervous system:** Hamada et al. (2009) cloned partial cDNA fragments of the
2 *Gryllus Dop1* and *Dop2* genes, and examined their mRNA distributions in the brain. They
3 reported that mRNAs of both dopamine receptors were expressed predominantly in the
4 Kenyon cells of the mushroom bodies in the cricket brain. In *Drosophila*, the DAMB (a
5 homologue of *Gryllus Dop2*) is exclusively expressed in the mushroom bodies (Han et al.,
6 1996), in which the dopaminergic system plays essential roles in learning and memory
7 (Waddell, 2010). In the brain of the honeybee and silkworm, mRNAs of dopamine receptors
8 are also detected in the Kenyon cells of the mushroom bodies (Kurshan et al., 2003; Kyle et
9 al., 2005; Mitumasu et al., 2008). In the cricket, pharmacological inhibition of D₁-like
10 dopamine receptor impaired aversive olfactory and visual conditioning and memory recall
11 (Unoki et al., 2005; Unoki et al., 2006; Mizunami et al., 2009). In *Drosophila*, the D₁-like
12 dopamine receptor DopR (a homologue of *Gryllus Dop1*) regulates caffeine-induced arousal
13 in the mushroom bodies (Andretic et al., 2008), as well as stress-induced arousal in the
14 central complex and sleep-wake arousal in the lateral-ventral neurons (Lebestky et al., 2009).
15 Moreover, the *DopEcR* gene is strongly expressed in the cricket CNS although its
16 physiological functions in the nervous system are still unknown in insects. Contrary to the
17 *Gryllus Dop1*, *Dop2* and *DopEcR* genes, which are expressed in all neuronal tissues
18 examined in this study, the *Gryllus* D₂-like dopamine receptor gene, *Dop3*, showed restricted
19 expression in the brain and SOG, and its expression was not detected in the optic lobes and
20 CC + CA. Draper et al. (2007) examined the distribution of the *Drosophila* D₂-like receptor
21 (DD2R) in the CNS using anti-DD2R antibodies, and found that a small number of neurons
22 including the Ap-let cohort of peptidergic neurons was immunoreactive in the larval and
23 adult CNS. They also generated DD2R RNA-interference (RNAi) lines and demonstrated that
24 the RNAi-mediated knock-down of the *DD2R* gene resulted in the reduction of locomotor
25 activity in the adult flies. On the other hand, in the honeybee, RNAi-mediated knockdown of

1 *AmDOP2* receptor gene affects locomotion (Mustard et al., 2010). Functional analysis of
2 each dopamine receptor in the cricket CNS will reveal the differential involvement of specific
3 dopamine receptor subtypes in behavior.

4 **Corpus cardiacum-corpora allata (CC-CA) complex:** The CC-CA complex functions as
5 an endocrine center in insects. Woodring and Hoffmann (1994) reported that dopamine
6 application has no effect on juvenile hormone biosynthesis in the isolated CA of adult
7 crickets. On the other hand, dopamine receptor blockers decreased release of adipokinetic
8 hormone (AKH) from the corpus cardiacum in the locust *Schistocerca gregaria*
9 (Samaranayaka, 1976). Dopamine stimulates cAMP accumulation in the CC of the cockroach
10 *Periplaneta americana* (Gole et al., 1987). In the CC of the locust *Locusta migratoria*,
11 dopamine potentiates cAMP-induced release of AKH, and is abundantly contained in the
12 storage part of the CC (Passier et al., 1995). Our study demonstrates that mRNAs of *Gryllus*
13 *Dop1* and *Dop2*, which are positively coupled to adenylyl cyclase, are expressed in the CC-
14 CA complex of adult crickets. Therefore, these receptors might be involved in the dopamine-
15 mediated stimulation of AKH release in the CC. In addition, the expression analysis revealed
16 that the *Gryllus DopEcR* gene, which mediates rapid response to ecdysteroids, was expressed
17 in the CC-CA complex of adult crickets. Our data suggest the presence of a feedback
18 regulation of the ecdysone system mediated by *DopEcR* in the CC-CA complex of the cricket.

19 **Salivary gland:** In insects, the aminergic and peptidergic systems control salivation (Ali,
20 1997; Walz et al. 2006). In *G. bimaculatus*, dopaminergic innervations in the salivary gland
21 originate from the SOG (SN1 neurons) (Helle et al., 1995; Hörner et al., 1995; Ali, 1997). In
22 the cockroach salivary gland, dopamine induces production of cAMP that acts as a second
23 messenger in the acinar cells to cause the secretory response (Grewe and Keabian, 1982;
24 Gray et al., 1984; Marg et al. 2004; Rietdorf et al. 2005). Our study demonstrates that
25 mRNAs of *Gryllus Dop1*, *Dop2* and *DopEcR*, which are positively coupled with adenylyl

cyclase, are expressed in the cricket salivary gland. Therefore, these receptors might be involved in the dopamine-mediated stimulation of salivation in the salivary gland.

Testicle and ovaries: Gonadotropic effect of dopamine has been reported in several insects. In the cockroach *Blattella germanica*, dopamine stimulates oocyte growth just before vitellogenesis, whereas it has an inhibitory effect at the end of vitellogenesis (Pastor et al., 1991). In *Drosophila*, depletion of dopamine in newly eclosed female flies resulted in abnormal development of the ovaries (Neckameyer, 1996). Gonadotropic effect of dopamine was also demonstrated in eusocial hymenoptera such as *Polistes chinensis* (Sasaki et al., 2009) and *Apis mellifera* (Dombroski et al., 2003). In *A. mellifera*, *AmDop1* and *AmDop3* are expressed in the worker ovaries, and their expression is associated with the reproductive status of workers (Vergoz et al., 2012). In contrast, in the cricket, *Gryllus Dop1*, *Dop2* and *DopEcR* genes are expressed in the ovaries, while the expression of the D₂-like dopamine receptor gene (*Dop3*) was not detected. We collected ovary samples from female crickets two weeks after adult molt. At this time the ovaries are fully activated. Like in *A. mellifera*, the expression of the dopamine receptor genes might be affected by the reproductive status in the cricket. To our knowledge, our study is the first report on the expression of dopamine receptor genes in the insect testicle. Our data suggest that the activity of both the female ovaries and the male testicle is regulated by dopamine in insects. Further histological and pharmacological studies are needed to understand the actions of dopamine on the testicle in the cricket.

Other non-neuronal tissues: *Gryllus Dop1* and *Dop2* genes were expressed in the midgut and Malpighian tubules. The catecholamine-containing nerve endings are present in the visceral muscle in insects (Klemm, 1972; Klemm, 1979). In *Locusta migratoria*, TH-like immunoreactive neurons are present in the stomatogastric nerve system, and dopamine inhibits phasic contraction of the foregut muscle (Lange and Chan, 2008). In *Drosophila*, the

D₂-like receptor is expressed in a small number of cells in the ventriculus and in the Malpighian tubules (Draper et al., 2007). In the cricket midgut and Malpighian tubules, we did not detect the expression of the D₂-like receptor gene by RT-PCR, but the expression of the *DopEcR* gene was detected in the midgut of adult crickets. Further studies are necessary to elucidate functional roles of dopamine receptor subtypes expressed in the cricket visceral organs. Contrary to the visceral muscle, little is known about the dopaminergic modulation of skeletal muscle activity in insect. In *Drosophila* larval neuromuscular junctions, dopamine reduces presynaptic activity but does not affect the postsynaptic receptiveness to glutamate (Cooper and Neckameyer, 1999). In the thoracic muscles of the cricket, the two D₁-like dopamine receptor genes and the *DopEcR* gene are expressed; therefore, dopamine can affect muscular activity *in vivo*. Further investigation is necessary to elucidate the dopaminergic control of skeletal muscle activity in the cricket.

In the present study, we determined the full-length coding sequence of the *Gryllus DAT* gene and examined tissue-specific distribution of the gene. Interestingly, our expression analysis revealed that the *Gryllus DAT* gene is ubiquitously expressed in the all cricket tissues examined in this study. In the other insects such as *Drosophila* and moths, it is reported that the *DAT* gene is predominantly expressed in the nervous tissues. In *Drosophila*, the *DAT* gene is detected in the brain and thoracic-abdominal ganglion but not in the other tissues (FlyAtlas Anatomical Expression Data; Chintapalli et al., 2007). In the larva of the cabbage looper moth *Trichoplusia ni*, Northern blot analysis revealed that mRNA of *DAT* gene was contained in the head (including the brain and SOG) but not in the fat body and epidermal tissues (Gallant et al., 2003). Further investigation is necessary to elucidate the physiological function of the *DAT* gene expressed in the non-neuronal tissues in the cricket.

1 In summary, we identified six genes involved in the biosynthesis, transduction and re-
2 uptake of dopamine in the cricket *G. bimaculatus*. Two TH isoforms showed isoform-specific
3 distribution in cricket tissues. Tissue-specific expression analysis of dopamine receptor genes
4 showed that the dopaminergic system is widely distributed in the cricket, and that the
5 dopaminergic system might regulate various aspects of physiological phenomena via distinct
6 dopamine receptor pathways.

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FIGURES AND TABLES

Figure 1

Comparison of the amino acid sequences of *Gryllus* TH with other TH proteins. (A) Alignment of the deduced amino acid sequence of *Gryllus* TH-A isoform with that of *Drosophila* pale-A isoform (NP_476897), *Mythimna* TH-A (type 2, brain form; BAF32574) and *Apis* TH (NP_001011633). Identical amino acids are printed in white letters on a black background. The catalytic domain is surrounded by a gray box. Black lines indicate the N-terminal regulatory (ACT) domain and the C-terminal coiled-coil region. The tetrahydrobiopterin (BH₄)-binding sites and iron binding sites are indicated by white and black arrowheads, respectively. The putative phosphorylation sites conserved between *Gryllus* TH and *Drosophila* pale proteins are indicated by white circles above the alignments. Two arginine residues important for catecholamine-mediated inhibition of enzyme activity (Nakashima et al., 1999; Nakashima et al., 2000) are indicated by black circles above the alignments. (B) Alignment of the deduced amino acid sequence of the ACT domain of insect TH-A and -B isoforms. GenBank accession numbers of aligned insect TH isoforms are as follows: *Drosophila* pale-A (NP_476897), *Drosophila* pale-B (NP_476898) *Mythimna* TH-B (BAF32573).

Figure 2

Molecular phylogenetic tree of *Gryllus* TH and other aromatic amino acid hydroxylases. The scale bar indicates 0.08 substitutions per site. The species name and the GenBank accession numbers of aromatic amino acid hydroxylases are as follows: *Drosophila* pale-A (*Drosophila melanogaster*, NP_476897), *Mythimna* TH (*Mythimna separata*, BAF32574), *Apis* TH (*Apis mellifera*, NP_001011633), *Tribolium* TH (*Tribolium castaneum*, NP_001092299), *Caenorhabditis* CAT-2 (*Caenorhabditis elegans*, ADZ54165), *Mus* TH

(*Mus musculus*, NP_033403), *Danio* TH (*Danio rerio*, NP_571224), *Danio* TH2 (*Danio rerio*, NP_001001829), *Drosophila* dTRH (*Drosophila melanogaster*, NP_612080), *Apis* TRH (*Apis mellifera*, XP_394674), *Tribolium* TRH (*Tribolium castaneum*, XP_967413), *Gryllus* TRH (*Gryllus bimaculatus*, BAJ83476), *Caenorhabditis* tph-1 (*Caenorhabditis elegans*, NP_495584), *Aplysia* TPH (*Aplysia californica*, ABF18968), *Mus* TpH1 (*Mus musculus*, NP_033440), *Danio* TpH1 (*Danio rerio*, NP_840091), *Mus* TpH2 (*Mus musculus*, NP_775567), *Danio* TpH2 (*Danio rerio* NP_999960), *Danio* tphd2 (*Danio rerio*, AAT38217), *Drosophila* Henna (*Drosophila melanogaster*, NP_523963), *Apis* TPH (*Apis mellifera*, XP_623300), *Tribolium* TPH (*Tribolium castaneum*, XP_967025), *Gryllus* TPH (*Gryllus bimaculatus*, BAJ83477), *Caenorhabditis* pah-1 (*Caenorhabditis elegans*, NP_495863), *Mus* TPH (*Mus musculus*, NP_032803), *Danio* TPH (*Danio rerio*, NP_956845).

Figure 3

Tissue-specific expression patterns of TH isoforms. The cDNA fragments corresponding to the catalytic domain (TH common region) and the isoform-specific ACT domains (TH-A isoform and TH-B isoform) were amplified by RT-PCR from ten cricket tissues. *Gryllus Ef1alpha* gene was amplified as an internal control gene. PCR products were run on a 1.5% agarose gel and stained with ethidium bromide. SOG = suboesophageal ganglion; CC + CA = corpus cardiacum-corpora allata complex.

Figure 4

Comparison of the amino acid sequences of *Gryllus* D₁-like dopamine receptor (Dop1) and INDR (Dop2) with *Apis* homologues. (A) Alignment of the deduced amino acid sequence of *Gryllus* Dop1 with that of *Drosophila* Dop1 (AAA85716), *Bombyx* Dop1 (NP_001108459) and *Apis* Dop1 (NP_001011595). (B) Alignment of the deduced amino acid

sequence of *Gryllus* Dop2 with that of *Drosophila* Dop2 (NP_733299), *Bombyx* Dop2 (NP_001108338) and *Apis* Dop2 (NP_001011567). Identical amino acids are printed in white letters on a black background. Black lines indicate the transmembrane segments (TM). The conserved DRY motif is indicated by white circles above the alignments. Putative N-glycosylation sites of *Gryllus* dopamine receptors are indicated by white arrowheads above the alignments. Putative phosphorylation sites conserved between dopamine receptors are indicated by black arrowheads under the alignments.

Figure 5

Molecular phylogenetic tree of *Gryllus* Dop1, Dop2 and other dopamine receptors. Vertebrate D₂-like dopamine receptors are used as an outgroup. The scale bar indicates 0.08 substitution per site. The species name and the GenBank accession numbers of the proteins are as follows: *Drosophila* Dop1 (*Drosophila melanogaster*, AAA85716), *Apis* Dop1 (*Apis mellifera*, NP_001011595), *Nasonia* Dop1 (*Nasonia vitripennis*, XP_001606438), *Acyrtosiphon* Dop1 (*Acyrtosiphon pisum*, XP_001947683), *Drosophila* Dop2 (*Drosophila melanogaster*, NP_733299), *Aedes* Dop2 (*Aedes aegypti*, XP_001651499), *Bombyx* Dop2 (*Bombyx mori*, NP_001108338), *Apis* Dop2 (*Apis mellifera*, NP_001011567), *Nasonia* Dop2 (*Nasonia vitripennis*, NP_001155849), *Aplysia* D₁-like (*Aplysia californica*, NP_001191631), *Danio* D₁ (*Danio rerio*, NP_001129448), *Danio* D₅-like 1 (*Danio rerio*, XP_001341592), *Danio* D₅-like 2 (*Danio rerio*, XP_692025), *Mus* D₁ (*Mus musculus*, NP_034206), *Mus* D₂ (*Mus musculus*, NP_034207), *Mus* D₃ (*Mus musculus*, NP_031903), *Mus* D₄ (*Mus musculus*, NP_031904), *Mus* D₅ (*Mus musculus*, NP_038531), *Mus* α_{1A} adrenergic receptor (*Mus musculus*, NP_038489), *Mus* α_{1B} adrenergic receptor (*Mus musculus*, NP_031442), *Danio* α_{1A} adrenergic receptor-like (*Danio rerio*, XP_001338938), *Danio* α_{1B} adrenergic receptor

(*Danio rerio*, NP_001007359), *Danio* α_{1B} adrenergic receptor-like (*Danio rerio*, XP_001922013), *Danio* α_{1D} adrenergic receptor (*Danio rerio*, XP_697043).

Figure 6

Comparison of the amino acid sequences of the *Gryllus* D₂-like dopamine receptor (Dop3) with other D₂-like dopamine receptors. (A) Alignment of the deduced amino acid sequence of *Gryllus* Dop3 with that of *Drosophila* DD2R (NP_001014760), *Apis* Dop3 (NP_001014983) and *Tribolium* Dop3 (XP_969037). Identical amino acids are printed in white letters on a black background. Black lines indicate the transmembrane segments (TM). (B) Molecular phylogenetic tree of *Gryllus* Dop3 and other dopamine receptors. Vertebrate D₁-like dopamine receptors are used as an outgroup. The scale bar indicates 0.07 substitutions per site. The species name and the GenBank accession numbers of the cDNAs are as follows: *Drosophila* DDR2 (*Drosophila melanogaster*, NM_001014760), *Apis* Dop3 (*Apis mellifera*, NM_001014983), *Nasonia* Dop3 (*Nasonia vitripennis*, XM_001602460), *Tribolium* Dop3 (*Tribolium castaneum*, XM_963944), *Pediculus* Dop3 (*Pediculus humanus corporis*, XM_002426878), *Panulirus* Dop3 (*Panulirus interruptus*, DQ900655), *Caenorhabditis* Dop-2 (*Caenorhabditis elegans*, NM_001028876), *Brugia* Dop-2 (*Brugia malayi*, XM_001901847), *Mus* D₁ (*Mus musculus*, NP_034206), *Mus* D₂ (*Mus musculus*, NM_010077), *Mus* D₃ (*Mus musculus*, NM_007877), *Mus* D₄ (*Mus musculus*, NM_007878), *Mus* D₅ (*Mus musculus*, NP_038531), *Danio* D_{2a} (*Danio rerio*, NM_183068), *Danio* D_{2b} (*Danio rerio*, NM_197936), *Danio* D₂-like (*Danio rerio*, NM_197935), *Danio* D₃ (*Danio rerio*, NM_183067), *Danio* D_{4a} (*Danio rerio*, NM_001012616), *Danio* D_{4b} (*Danio rerio*, NM_001012618), *Danio* D₄-related (*Danio rerio*, NM_001012620).

Figure 7

Comparison of the amino acid sequences of *Gryllus* DopEcR with other G-protein coupled receptors. (A) Alignment of the deduced amino acid sequence of *Gryllus* DopEcR with that of *Drosophila* DopEcR (NP_647897), *Apis* DopEcR (XP_396491) and *Tribolium* DopEcR (XP_968380). Identical amino acids are printed in white letters on a black background. Black lines indicate the transmembrane segments (TM). The DRY motif is indicated by white circles above the alignments. (B) Molecular phylogenetic tree of *Gryllus* DopEcR and other G-protein coupled receptors. *Mus musculus* histamine H₂ receptor is used as an outgroup. The scale bar indicates 0.15 substitutions per site. The species name and the GenBank accession numbers of the proteins are as follows: *Drosophila* DopEcR (*Drosophila melanogaster*, NP_647897), *Aedes* DopEcR (*Aedes aegypti*, XP_001654794), *Apis* DopEcR (*Apis mellifera*, XP_396491), *Tribolium* DopEcR (*Tribolium castaneum*, XP_968380), *Mus* GPR21 (*Mus musculus*, NP_001139802), *Gallus* GPR21 (*Gallus gallus*, XP_001233342), *Xenopus* GPR21 (*Xenopus tropicalis*, XP_002931480), *Mus* GPR52 (*Mus musculus*, NP_796357), *Gallus* GPR52 (*Gallus gallus*, XP_001234532), *Danio* GPR52 (*Danio rerio*, CAK04352), *Drosophila* OA β ₁ (*Drosophila melanogaster*, AJ880687), *Drosophila* OA β ₂ (*Drosophila melanogaster*, NP_001034049), *Mus* β ₁ adrenergic receptor (*Mus musculus*, NP_031445), *Mus* β ₂ adrenergic receptor (*Mus musculus*, NP_031446), *Mus* β ₃ adrenergic receptor (*Mus musculus*, NP_038490), *Mus* histamine H₂ receptor (*Mus musculus*, NP_032312).

Figure 8

Tissue specific expression patterns of mRNA of *Gryllus* dopamine receptor genes. The cDNA fragments of *Gryllus* *Dop1*, *Dop2*, *Dop3* and *DopEcR* genes were amplified by RT-PCR from ten cricket tissues. The *Gryllus* *Ef1alpha* gene was amplified as an internal control

gene. PCR products were run on a 1.5% agarose gel and stained with ethidium bromide. SOG = suboesophageal ganglion; CC + CA = corpus cardiacum-corpora allata complex.

Figure 9

Comparison of the amino acid sequences of *Gryllus* DAT with other monoamine transporters. (A) Alignment of the deduced amino acid sequence of *Gryllus* DAT with that of *Drosophila* DAT (NP_523763), *Bombyx* DAT (NP_001037362) and *Apis* DAT (NP_001139210). Identical amino acids are printed in white letters on a black background. Black lines indicate the transmembrane segments (TM). Putative N-glycosylation sites of *Gryllus* DAT are indicated by white arrowheads above the alignments. Putative phosphorylation sites conserved between *Gryllus* and *Drosophila* DAT are indicated by black arrowheads under the alignments. ‘S-S bond’ indicates two conserved cystine residues that form a disulfide bond (Chen et al., 2007). (B) Molecular phylogenetic tree of *Gryllus* DAT and other monoamine transporters. The scale bar indicates 0.06 substitutions per site. The species name and the GenBank accession numbers of the biogenic amine transporters are as follows: *Drosophila* DAT (*Drosophila melanogaster*, NP_523763), *Aedes* DAT (*Aedes aegypti*, XP_001654246), *Bombyx* DAT (*Bombyx mori*, NP_001037362), *Trichoplusia* DAT (*Trichoplusia ni*, AAN52844), *Apis* DAT (*Apis mellifera*, NP_001139210), *Caenorhabditis* dat-1 (*Caenorhabditis elegans*, NP_499043), *Mus* DAT (*Mus musculus*, NP_034150), *Danio* DAT (*Danio rerio*, NP_571830), *Mus* NAT (*Mus musculus*, NP_033235), *Danio* NAT (*Danio rerio*, XP_694138), *Trichoplusia* OAT (*Trichoplusia ni*, AF388173), *Tribolium* OAT (*Tribolium castaneum*, XP_975356), *Pediculus* OAT (*Pediculus humanus corporis*, XP_002425932), *Acyrtosiphon* OAT (*Acyrtosiphon pisum*, XP_001949303), *Drosophila* SERT (*Drosophila melanogaster*, NP_523846), *Bombyx* SERT (*Bombyx mori*, NP_001037436), *Manduca* SERT (*Manduca sexta*, AAN59781), *Tribolium* SERT (*Tribolium*

1 *castaneum*, XP_968717), *Acyrtosiphon* SERT (*Acyrtosiphon pisum*, XP_001944311), *Mus*
2 SERT (*Mus musculus*, NP_034614).

3

4 **Figure 10**

5 Tissue-specific expression pattern of *Gryllus* *DAT* gene. The cDNA fragments of
6 *Gryllus* *DAT* gene were amplified by RT-PCR from ten cricket tissues. *Gryllus* *Ef1alpha*
7 gene was amplified as an internal control gene. PCR products were run on a 1.5% agarose gel
8 and stained with ethidium bromide. SOG = suboesophageal ganglion; CC + CA = corpus
9 cardiacum-corpora allata complex.

1 **Table 1. List of primers used in this study**

Degenerate primers

	Forward primer	Reverse primer
<i>TH</i> (C-terminal region)	5'-	5'-CACYTCTGAANGGYCGSGACAT-3'
<i>DopEcR</i>	GGTNTAYACNTTYATGTGGAT -3'	5'- GRCCATCCARCAYTGRCA -3'
<i>DAT</i>	5'-GGCATHCCYYTNTTCTACATG G-3'	5'-ATDATGACGTTRTAGTAGAARTCNACG TA-3'

Primers for full-length/partial ORF amplification

	Forward primer	Reverse primer
<i>TH</i>	5'-CCGCGACAACAACCTCTCAG-3'	5'-CATGCCGGTGGACACCCAC-3'
<i>Dop1</i>	5'-GCGCCGCCATGGAGGACGACG-3'	5'-GCGGCCGTCTCTTCAGATGG-3'
<i>Dop2</i>	5'-GCCGACGAAACAACCTCGAG-3'	5'-CCGGCTGTTTGTTCAGGTGG-3'

<i>Dop3</i>	5'-GCCCTTCTTCACGTGCAAC- 3'	5'-CCAGCGCGTGTTGTTACG-3'
	5'-	
<i>DopEcR</i>	CCCTGCACCCCTCACCGCGAC	5'-CCAGCGCGTGTTGTTACG-3'
	TATG-3'	
		5'-
<i>DAT</i>	5'-CGCTGAGGAGCTGCAGATG- 3'	GAGCACCTTTTGGCAAATAGAGAAATC ACAC-3'

Primers for tissue-specific expression

analysis

	Forward primer	Reverse primer
<i>TH</i>		
common region	5'-GGCCTTCCGGATCTTCC-3'	5'-GGGTGTCCACCGGCATG-3'
<i>TH-A</i> isoform	5'-CCGCGACAACAACCTCTCAG-3'	5'-CCGTCAGCTCAGCGTCGTTG-3'
<i>TH-B</i> isoform	5'-CCGCGACAACAACCTCTCAG-3'	5'-CCGTCAGCTCAGCGTCATC-3'
<i>Dop1</i>	5'-CGCCGTACCACGTGTCTG-3'	5'-CGCCGAGTTGGAGTAGC-3'
<i>Dop2</i>	5'-CCAACTCCAGCATGAATCC-3'	5'-GAGTGCGCCAGCATCATGG-3'

Dop3 5'-GCCATGTGCACCAAGCTG-3' 5'-CCAGCGCGTGTTGTTACG-3'

5'-

DopEcR CGTACAGCGGTGGGTGTATGG 5'-CGTCTGCACCGTCTCGTAGC-3'

-3'

5'-

5'-

DAT GTGGCGAGATCATCAAATAGC GAGCACCTTTTGGCAAATAGAGAAATC

TGC-3'

ACAC-3'

5'-

Ef1alpha GTGTTCTGAAGCCAGGTATGG-5'-CTCCAGCAACATAACCACGAC-3'

3'

A

Gryllus TH-A 1 MMVAAAAQKNREMPAIIKSSYSIENGYPARRRSVVDDARFETLVVKQTKQSALDEARQSSNDALSTEEVLLANAASSSSDAPAAQSAALVLRRL
Drosophila pale-A 1 MMVAAAAQKNREMPAIIKSSYSIENGYPARRRSVVDDARFETLVVKQTKQSVLEEARANDVLTEDDELLANAASSSSDAPAAQSAALVLRRL
Mythimna TH-A 1 MMVAAAAQKNREMPAIIKSSYSIENGYPARRRSVVDDARFETLVVKQTKQSVLEEARANDVLTEDDELLANAASSSSDAPAAQSAALVLRRL
Apis TH 1 MMVAAAAQKNREMPAIIKSSYSIENGYPARRRSVVDDARFETLVVKQTKQSVLEEARORANDALGTEEEVVLAKTIACPESENTVQAAALVLRRL

Regulatory (ACT) domain

Gryllus TH-A 96 DGVGALARILKTIENFKGSVTHVEHRAAGTGARADALVRVDISROQLLLLRALRQSAALSAVALLADTHVSIKDPWFFRHASLDNCNHLMTK
Drosophila pale-A 96 EGISGLGRILKAIETHTVTHVESRQSRVGEVDHDLVRKDDMRGNLLQLIRSLRQSGSFSSNNMLADNNLNVPKAPWFFRHASLDNCNHLMTK
Mythimna TH-A 95 DGMGSLARILKTIENFKGVQVHETRPQSOLTGVQFDALVKVSMGRINLLQLIRSLRQSTSFACVNLVSENNHSSRTPWFFRHASLDNCNHLMTK
Apis TH 96 EGISGLARILKTIENFKGTVTHVESRPSRKKEGQFDLVKVVMDRQVLLQLIRNLRSLSALDGVLLADNSVISIKDPWFFRHASLDNCNHLMTK

Gryllus TH-A 191 YEPFLDMNHHPGFADKVVYRERRKMTADIAFAFRKGGPIPTTGYAAATATWVVFNNVDFLPHACAEYRRVCGHCEANIFCPRIPODEMS
Drosophila pale-A 191 YEPFLDMNHHPGFADKVVYRERRKMTADIAFAFRKGGPIPTTGYAAATATWVVFNNVDFLPHACAEYRRVCGHCEANIFCPRIPODEMS
Mythimna TH-A 190 YEPFLDMNHHPGFADKVVYRERRKMTADIAFAFRKGGPIPTTGYAAATATWVVFNNVDFLPHACAEYRRVCGHCEANIFCPRIPODEMS
Apis TH 191 YEPFLDMNHHPGFADKVVYRERRKMTADIAFAFRKGGPIPTTGYAAATATWVVFNNVDFLPHACAEYRRVCGHCEANIFCPRIPODEMS

Catalytic domain

Gryllus TH-A 286 FLNRHGTGFLRPAAGLLTARDFLASLAFRNFQSTQYVHRHSSSPHTPEPPDCIHEMIGHMPLLADPFAQFSQIEGLASLGASDAIEIKLSTVYWF
Drosophila pale-A 286 FLRRNGTSLRPAAGLLTARDFLASLAFRNFQSTQYVHRHNSPYHTPEPPSIEHLLGHMPLLADPSFAQFSQIEGLASLGASDAIEIKLSTVYWF
Mythimna TH-A 285 FLRRHGTGFLRPAAGLLTARDFLASLAFRNFQSTQYVHRHNSPYHTPEPPDCIHELLGHPLLADPSFAQFSQIEGLASLGASDAIEIKLSTVYWF
Apis TH 286 FLRRNGTGLTLPAPAGLLTARDFLASLAFRNFQSTQYVHRHNSPYHTPEPPDCIHELLGHMPLLADPSFAQFSQIEGLASLGASDAIEIKLSTVYWF

Gryllus TH-A 381 RVEFGLCCKEHLKAYGAGLLSSVYGLLHALSDQCHRRPEPALTAQVQYODQYQPIYVVAESFEDAKDKFRWVSTGM-----
Drosophila pale-A 381 RVEFGLCCKEHLKAYGAGLLSSVYGLLHALSDQCHRRPEPALTAQVQYODQYQPIYVVAESFEDAKDKFRWVSTGM-----
Mythimna TH-A 380 RVEFGLCCKEHLKAYGAGLLSSVYGLLHALSDQCHRRPEPALTAQVQYODQYQPIYVVAESFEDAKDKFRWVSTMRPPFVRFNPHFRV
Apis TH 381 RVEFGLCCKEGLDYLKAYGAGLLSSVYGLLHALSDQCHRRPEPKSTAVQVQYQDQYQPIYVVAESFEDAKDKFRWVSTMRPPFVRFNPHFRV

Gryllus TH-A 475 EVLDSVDKLETLVHOMNTEHLHLTNALSKRRP-
Drosophila pale-A 474 EVLDSVDKLETLVHOMNTEHLHLTNALSKRRP-
Mythimna TH-A 474 EVLDSVDKLETLVHOMNTEHLHLTNALSKRRP-
Apis TH 476 EVLDSVDKLETLVHOMNTEHLHLTNALSKRRP-

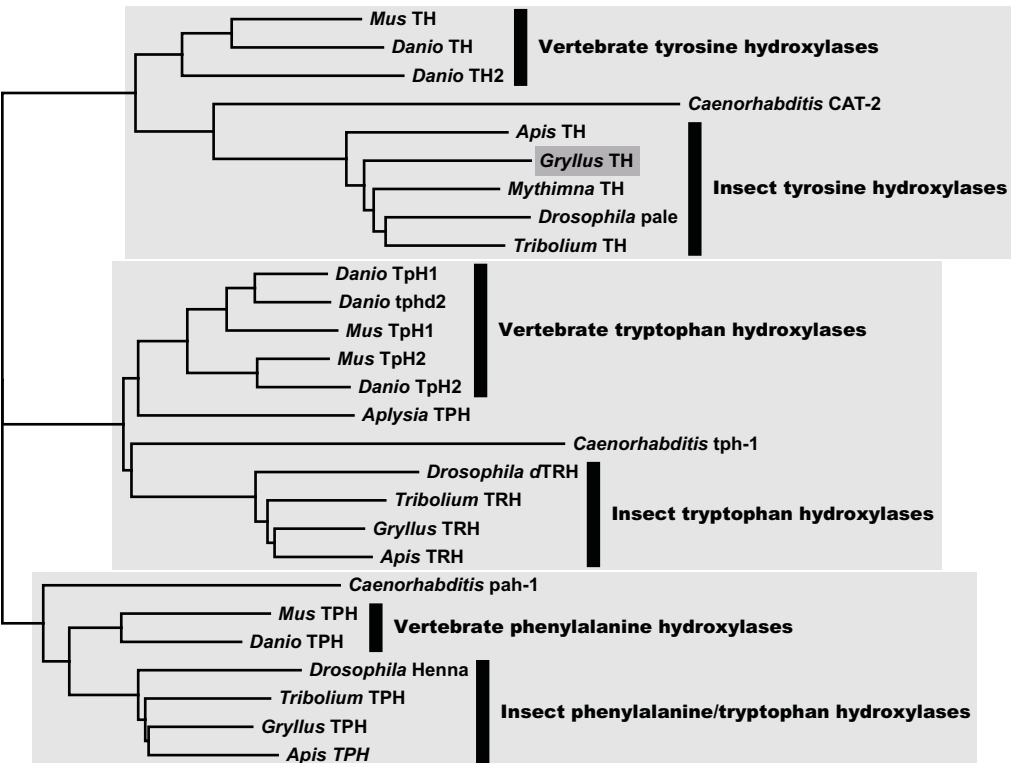
Coiled coil region**B**

Gryllus TH-A 29 RRSVVDDARFETLVVKQTKQSALDEARQSSNDALSTEEVLLANAASSSSDAPAAQSAALVLRRL
Mythimna TH-A 30 RRSVVDDARFETLVVKQTKQSVLEEARANDVLTEDDELLANAASSSSDAPAAQSAALVLRRL
Drosophila pale-A 30 RRSVVDDARFETLVVKQTKQSVLEEARANDVLTEDDELLANAASSSSDAPAAQSAALVLRRL
Gryllus TH-B 30 RRSVVDDARFETLVVKQTKQSALDEARQSSNDALSTEEVLLANAASSSSDAPAAQSAALVLRRL
Mythimna TH-B 29 RRSVVDDARFETLVVKQTKQSALDEARQSSNDALSTEEVLLANAASSSSDAPAAQSAALVLRRL
Drosophila pale-B 30 RRSVVDDARFETLVVKQTKQSVLEEARANDVLTEDDELLANAASSSSDAPAAQSAALVLRRL

B isoform-specific insertion in the ACT domain

Gryllus TH-A 61 -----DALLTEPEMILTATARAASBAEAQAQVQAALVLRRLDVGAGARILKLETFKCSVTVVRAAAGKGVQ
Mythimna TH-A 60 -----DYLTEPEMILTQNAASSSPEAQAQVQAALVLRRLDVGAGARILKLETFKCSVTVVRAAAGKGVQ
Drosophila pale-A 61 -----DGLTEPEMILTANAASSSPEAQAQVQAALVLRRLDVGAGARILKLETFKCSVTVVRAAAGKGVQ
Gryllus TH-B 107 PEPFPPDPTPEQL- - - - -DALLTEPEMILTATARAASBAEAQAQVQAALVLRRLDVGAGARILKLETFKCSVTVVRAAAGKGVQ
Mythimna TH-B 98 -----ADADIGD-DAG- - - - -DGLTEPEMILTQNAASSSPEAQAQVQAALVLRRLDVGAGARILKLETFKCSVTVVRAAAGKGVQ
Drosophila pale-B 108 - - - - -SVQPEQESQSQPE- - - - -GNOQPTKMDYLTEPEMILTANAASSSPEAQAQVQAALVLRRLDVGAGARILKLETFKCSVTVVRAAAGKGVQ

Gryllus TH-A 130 ADAVVRVD
Mythimna TH-A 129 FDAVVKVS
Drosophila pale-A 130 HDVIRKLD
Gryllus TH-B 196 ADAVVRVD
Mythimna TH-B 181 FDAVVKVS
Drosophila pale-B 201 HDVIRKLD



Central brain	Lamina + Medulla + Retina	Thoracic muscles	Malpighian tubules
SOG	CC + CA	Salivary gland	Testicle
		Midgut	Ovary

1990 1991 1992 1993 1994 1995 1996 1997 1998 1999 2000 2001 2002 2003 2004 2005 2006 2007 2008 2009 2010 2011 2012 2013 2014 2015 2016 2017 2018 2019 2020 2021 2022 2023 2024 2025 2026 2027 2028 2029 2030 2031 2032 2033 2034 2035 2036 2037 2038 2039 2040 2041 2042 2043 2044 2045 2046 2047 2048 2049 2050 2051 2052 2053 2054 2055 2056 2057 2058 2059 2060 2061 2062 2063 2064 2065 2066 2067 2068 2069 2070 2071 2072 2073 2074 2075 2076 2077 2078 2079 2080 2081 2082 2083 2084 2085 2086 2087 2088 2089 2090 2091 2092 2093 2094 2095 2096 2097 2098 2099 2100 2101 2102 2103 2104 2105 2106 2107 2108 2109 2110 2111 2112 2113 2114 2115 2116 2117 2118 2119 2120 2121 2122 2123 2124 2125 2126 2127 2128 2129 2130 2131 2132 2133 2134 2135 2136 2137 2138 2139 2140 2141 2142 2143 2144 2145 2146 2147 2148 2149 2150 2151 2152 2153 2154 2155 2156 2157 2158 2159 2160 2161 2162 2163 2164 2165 2166 2167 2168 2169 2170 2171 2172 2173 2174 2175 2176 2177 2178 2179 2180 2181 2182 2183 2184 2185 2186 2187 2188 2189 2190 2191 2192 2193 2194 2195 2196 2197 2198 2199 2200 2201 2202 2203 2204 2205 2206 2207 2208 2209 2210 2211 2212 2213 2214 2215 2216 2217 2218 2219 2220 2221 2222 2223 2224 2225 2226 2227 2228 2229 2230 2231 2232 2233 2234 2235 2236 2237 2238 2239 2240 2241 2242 2243 2244 2245 2246 2247 2248 2249 2250 2251 2252 2253 2254 2255 2256 2257 2258 2259 2260 2261 2262 2263 2264 2265 2266 2267 2268 2269 2270 2271 2272 2273 2274 2275 2276 2277 2278 2279 2280 2281 2282 2283 2284 2285 2286 2287 2288 2289 2290 2291 2292 2293 2294 2295 2296 2297 2298 2299 2300 2301 2302 2303 2304 2305 2306 2307 2308 2309 2310 2311 2312 2313 2314 2315 2316 2317 2318 2319 2320 2321 2322 2323 2324 2325 2326 2327 2328 2329 2330 2331 2332 2333 2334 2335 2336 2337 2338 2339 2340 2341 2342 2343 2344 2345 2346 2347 2348 2349 2350 2351 2352 2353 2354 2355 2356 2357 2358 2359 2360 2361 2362 2363 2364 2365 2366 2367 2368 2369 2370 2371 2372 2373 2374 2375 2376 2377 2378 2379 2380 2381 2382 2383 2384 2385 2386 2387 2388 2389 2390 2391 2392 2393 2394 2395 2396 2397 2398 2399 2400 2401 2402 2403 2404 2405 2406 2407 2408 2409 2410 2411 2412 2413 2414 2415 2416 2417 2418 2419 2420 2421 2422 2423 2424 2425 2426 2427 2428 2429 2430 2431 2432 2433 2434 2435 2436 2437 2438 2439 2440 2441 2442 2443 2444 2445 2446 2447 2448 2449 2450 2451 2452 2453 2454 2455 2456 2457 2458 2459 2460 2461 2462 2463 2464 2465 2466 2467 2468 2469 2470 2471 2472 2473 2474 2475 2476 2477 2478 2479 2480 2481 2482 2483 2484 2485 2486 2487 2488 2489 2490 2491 2492 2493 2494 2495 2496 2497 2498 2499 2500 2501 2502 2503 2504 2505 2506 2507 2508 2509 2510 2511 2512 2513 2514 2515 2516 2517 2518 2519 2520 2521 2522 2523 2524 2525 2526 2527 2528 2529 2530 2531 2532 2533 2534 2535 2536 2537 2538 2539 2540 2541 2542 2543 2544 2545 2546 2547 2548 2549 2550 2551 2552 2553 2554 2555 2556 2557 2558 2559 2560 2561 2562 2563 2564 2565 2566 2567 2568 2569 2570 2571 2572 2573 2574 2575 2576 2577 2578 2579 2580 2581 2582 2583 2584 2585 2586 2587 2588 2589 2590 2591 2592 2593 2594 2595 2596 2597 2598 2599 2600 2601 2602 2603 2604 2605 2606 2607 2608 2609 2610 2611 2612 2613 2614 2615 2616 2617 2618 2619 2620 2621 2622 2623 2624 2625 2626 2627 2628 2629 2630 2631 2632 2633 2634 2635 2636 2637 2638 2639 2640 2641 2642 2643 2644 2645 2646 2647 2648 2649 2650 2651 2652 2653 2654 2655 2656 2657 2658 2659 2660 2661 2662 2663 2664 2665 2666 2667 2668 2669 2670 2671 2672 2673 2674 2675 2676 2677 2678 2679 2680 2681 2682 2683 2684 2685 2686 2687 2688 2689 2690 2691 2692 2693 2694 2695 2696 2697 2698 2699 2700 2701 2702 2703 2704 2705 2706 2707 2708 2709 2710 2711 2712 2713 2714 2715 2716 2717 2718 2719 2720 2721 2722 2723 2724 2725 2726 2727 2728 2729 2730 2731 2732 2733 2734 2735 2736 2737 2738 2739 2740 2741 2742 2743 2744 2745 2746 2747 2748 2749 2750 2751 2752 2753 2754 2755 2756 2757 2758 2759 2760 2761 2762 2763 2764 2765 2766 2767 2768 2769 2770 2771 2772 2773 2774 2775 2776 2777 2778 2779 2780 2781 2782 2783 2784 2785 2786 2787 2788 2789 2790 2791 2792 2793 2794 2795 2796 2797 2798 2799 2800 2801 2802 2803 2804 2805 2806 2807 2

A Western blot image showing several horizontal bands of varying intensity across approximately ten lanes. The bands represent different protein levels or isoforms detected by the antibody used.

RT(+)
RT(-)

2008 2009 2010 2011 2012 2013 2014 2015 2016 2017

A

Gryllus Dopl1 1 MTNMAIRAIAAAGVGSAATVATSTTSSISSSTTIINTSSATTIGGNHTSGSTGFSTNSTLLDADHLPQLTTAKVDLDEIDIQLLTNGYDGT ME-
Drosophila Dopl1 1 MTNMAIRAIAAAGVGSAATVATSTTSSISSSTTIINTSSATTIGGNHTSGSTGFSTNSTLLDADHLPQLTTAKVDLDEIDIQLLTNGYDGT ME-FAST
Bombyx Dopl1 1
Apis Dopl1 1

Gryllus Dopl1 8 VNG---SSASAGPSGGAGGASADSNDGDLSTSPVGVVLCALLSLILFLSHAGNVLVCVAIYTRGLRRRIGNLFLASLAADLVFAALVMTFAV
Drosophila Dopl1 96 TILTSFYNESSMTNASEMTITGGEPEPLSHVGVVLCALLSLILFLSHAGNVLVCVAIYTRGLRRRIGNLFLASLAADLVFAALVMTFAV
Bombyx Dopl1 7 ---LAE---NVEPLIDAEVVDGDAVLGLHVLGLLILFLSHAGNVLVCVAIYTRGLRRRIGNLFLASLAADLVFAALVMTFAV
Apis Dopl1 1 -----MILSONNITDQKEDDPENTFSLSLVGVVLCALLSLILFLSHAGNVLVCVAIYTRGLRRRIGNLFLASLAADLVFAALVMTFAV

oco TM1 TM2
Gryllus Dopl1 100 NDLLGHVDFGEALCDTWAFDVMCSTASILNLCAISLDRIYHIKDPRLRYGRWVRRVAVGSIATVWLLAALVSFVPISLGLHRPDEPPPAARG
Drosophila Dopl1 188 NDLLGVHDFGEALCDTWAFDVMCSTASILNLCAISLDRIYHIKDPRLRYGRWVRRVAVGSIATVWLLAALVSFVPISLGLHRPDEPPPAARG
Bombyx Dopl1 95 NDLLGVHDFGEALCDTWAFDVMCSTASILNLCAISLDRIYHIKDPRLRYGRWVRRVAVGSIATVWLLAALVSFVPISLGLHRPDEPPPAARG
Apis Dopl1 85 NDLLGVHDFGEALCDTWAFDVMCSTASILNLCAISLDRIYHIKDPRLRYGRWVRRVAVGSIATVWLLAALVSFVPISLGLHRPDEPPPAARG

TM3 TM4
Gryllus Dopl1 195 PRGEBELTCCALDLAPFYAVSSGVSTPCLVHVIYCYRILYIAQKHKVSRATVTFWGMG-----AGPESHVGV-----SQAGSSPH
Drosophila Dopl1 279 DKGKVEHETCCALDLAPFYAVSSGVSTPCLVHVIYCYRILYIAQKHKVSRATVTFWGMG-----AGPESHVGV-----SQAGSSPH
Bombyx Dopl1 186 ODFPKPYTCALDLAPFYAVSSGVSTPCLVHVIYCYRILYIAQKHKVSRATVTFWGMG-----AGPESHVGV-----SQAGSSPH
Apis Dopl1 176 DSKVEHETCCALDLAPFYAVSSGVSTPCLVHVIYCYRILYIAQKHKVSRATVTFWGMG-----AGPESHVGV-----SQAGSSPH

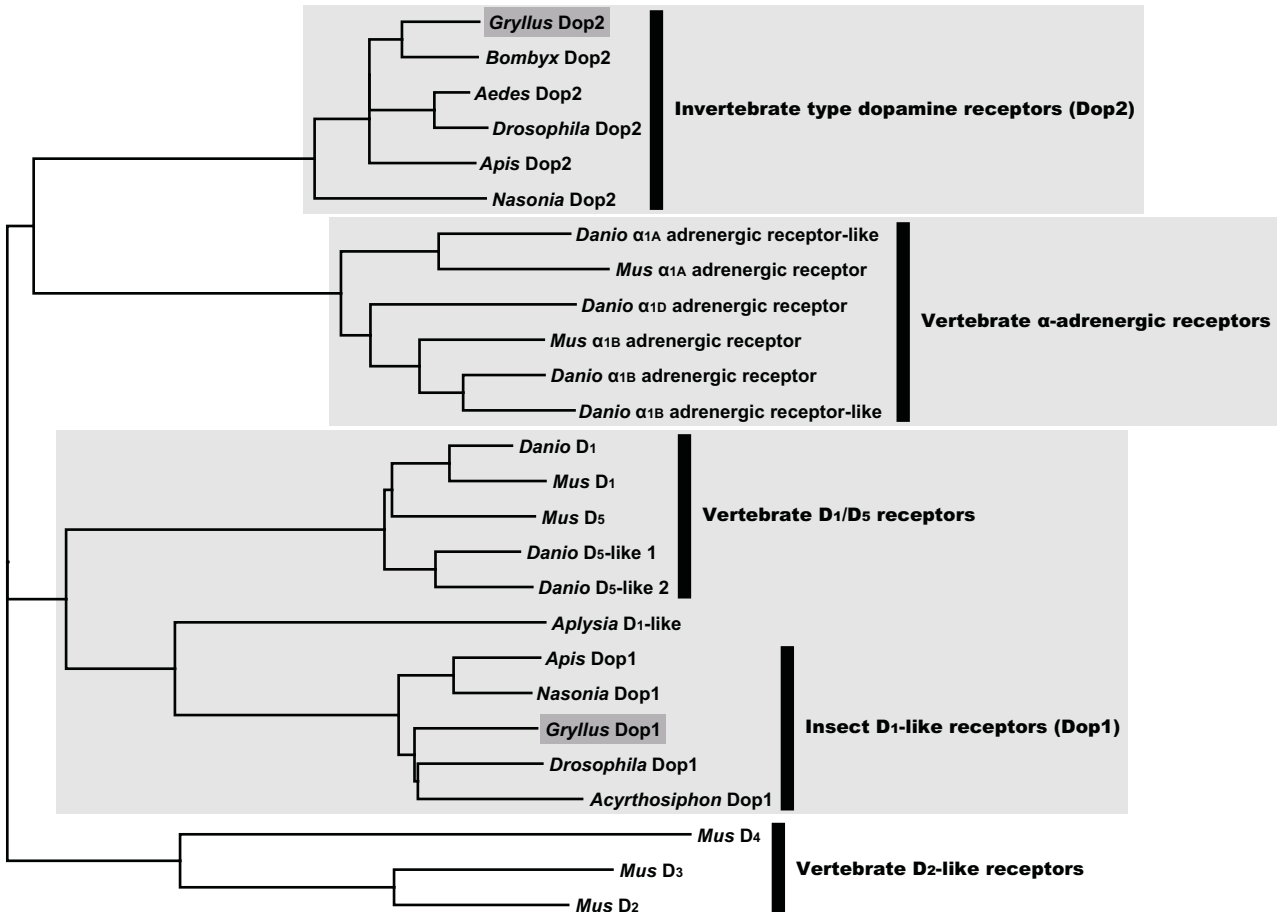
TM5 TM6 TM7
Gryllus Dopl1 279 VSDHKAATVGVINGVFLVCVWVFFCVNIHAAPFKCTICPDAFRVLITWLGYNSAFNPYIYSIFNKEFRDAFNRILANAAVDPCCGCCPAC
Drosophila Dopl1 321 VSDHKAATVGVINGVFLVCVWVFFCVNIHAAPFKCTICPDAFRVLITWLGYNSAFNPYIYSIFNKEFRDAFNRILANAAVDPCCGCCPAC
Bombyx Dopl1 268 VSDHKAATVGVINGVFLVCVWVFFCVNIHAAPFKCTICPDAFRVLITWLGYNSAFNPYIYSIFNKEFRDAFNRILANAAVDPCCGCCPAC
Apis Dopl1 265 VSDHKAATVGVINGVFLVCVWVFFCVNIHAAPFKCTICPDAFRVLITWLGYNSAFNPYIYSIFNKEFRDAFNRILANAAVDPCCGCCPAC

Gryllus Dopl1 372 GEFVSSRSLSLAAGGGRS-KASVDCGLGGMGAGAGCGGGGACAAADAV-----TPRSSAGAAAAVPTAVHASKPPPPQSPAQPPPPSP
Drosophila Dopl1 456 -GDV-GNTH-----RNSDRFIDYGA-----RNVVV-MI-GS-----RSSA
Bombyx Dopl1 352 GTCGVNTH-P-----RNDFFVDYGA-----RNVVV-MI-GS-----RSSA
Apis Dopl1 344 -A-RARGNP-----STSCGE-----FRSVVVKI-----GSM-TECNI-----SPRSSACQVGM

Gryllus Dopl1 463 QPRLAETRVDSDEDP-EEEVSAT
Drosophila Dopl1 489 -----LEQ-----VSAT
Bombyx Dopl1 402 -----YH-----VSAT
Apis Dopl1 391 -----AQRH-----VSAT

B

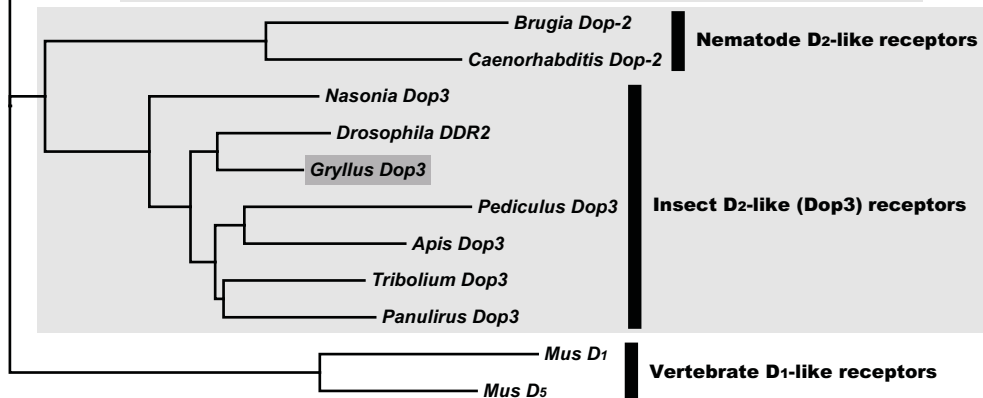
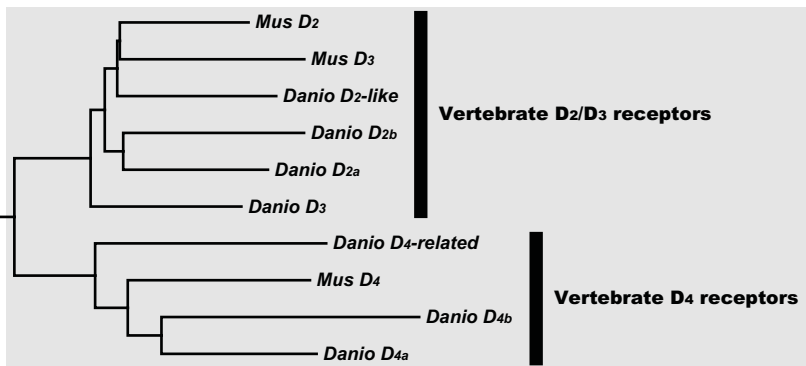
Figure 10. Sequence logo for the 100 amino acid motif. The y-axis represents information content in bits, ranging from 0 to 1.5. The x-axis shows the 20 amino acids. The most conserved position is the 10th, with a strong preference for Aspartate (D) and Glutamate (E). Other positions show varying degrees of conservation, with the 1st position being highly conserved for Aspartate (D) and Glutamate (E).



A

<i>Gryllus</i> Dop3	1	PFFTCNIMDAMCTKLSLDCS	PGVTAFILTTWLGYNMNSFVNPIVYIT	INPEFRKAFKKLMALG	T
<i>Drosophila</i> DDR2	545	PPFSCNIMDAMCTKFKKDCRPG	LTAYMTTWLGV	INNSFVNPIVYIT	FNPEFRKAFKKLMHMC
<i>Apis</i> Dop3	634	PPFSCNIMDAMCTKLTAD	COPGVTAFTVTSWLGYNMNSFVNPIVYIT	FNPEFRKAFHKLVSF	--
<i>Tribolium</i> Dop3	391	PPFTCNIMDAMCSKLN	PCOPGVAAFTLTWLGYNMNSFVNPIVYIT	FNPEFRKAFKKLMFLGP	

TM6 TM7

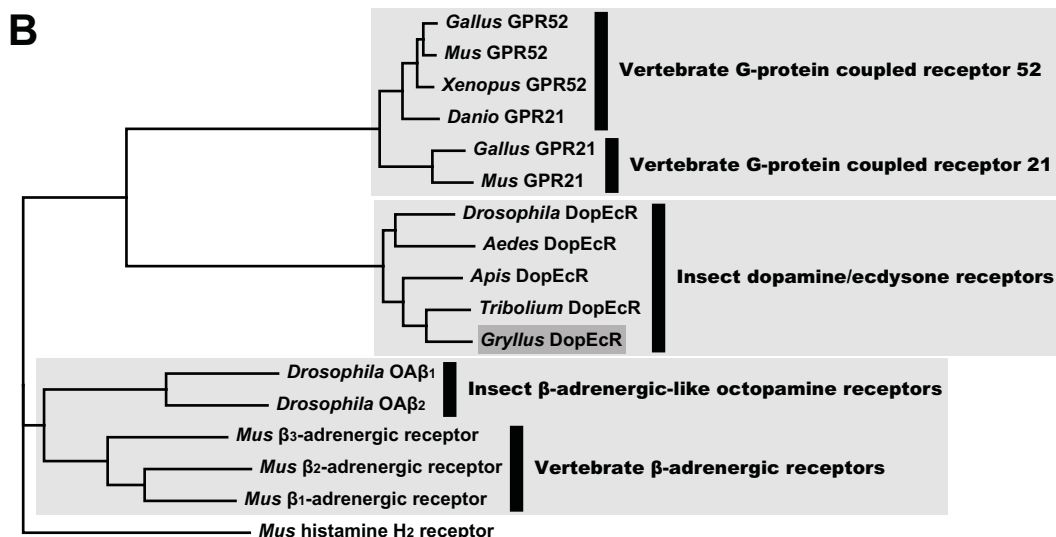
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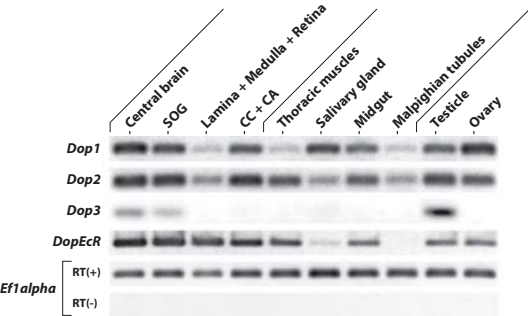
0.07

A

Species	Accession	Position	Sequence
<i>Gryllus</i>	DopEcR	1	-----MIVLSFDNDHNTREFTLELTOAGHFPVPGVAIVVNVLIATLNFPGPSEVNVLLSLAVADLLCGLLVLPVLSVYPALVQRN
<i>Drosophila</i>	DopEcR	1	-----MQMSXYLDNS-----KVEALRAVLISGTGVAIVLSNLIATANFGPPEVENVYLLSLAADDLLCGLLVVPFVSYPATGVRS
<i>Apis</i>	DopEcR	1	-----MEEP-----SLEALTOAGHFPVPGVAIVLSNLIATLNFPGPSEVNVYLLSLAADDLLCGLLVLPVLSVYPALVQRN
<i>Tribolium</i>	DopEcR	1	MFRTRDSTSAHTIFSDNSNDGIVFTLELCTOAGHFPVPGVAIVVNVLIATLNFPGPSEVNVLLSLAVADLLCGLLVLPVLSVYPALVQRN
			TM1
			TM2
<i>Gryllus</i>	DopEcR	86	VYGDVVMCRIVGYLEVLTWAVSVVFFMWNISVDRYLAVRKPLRYETVQTKRCQCWMVFTWISAAMLCPPPLGPNHFFD-RDAVICMLDWGMMAA
<i>Drosophila</i>	DopEcR	82	VYGDIVCRTEGYLEVLTWAVSVVFFMWNISVDRYLAVRKPLRYETVQTKRCQCWMVFTWISAAMLCPPPLGYSMPFENNTHICMLDWGMMAA
<i>Apis</i>	DopEcR	75	VYGDIVCRIVGYLEVLTWAVSVVFFMWNISVDRYLAVRKPLRYETVQTKRCQCWMVFTWISAAMLCPPPLGFKRPIFD-REAAICMLDWGMMAA
<i>Tribolium</i>	DopEcR	96	VYGDIVCRIVGYLEVLTWAVSVVFFMWNISVDRYLAVRKPLRYETVQTKRCQCWMVFTWISAAMLCPPPLGFGVFFD-RDAVICMLDWGMMAA
			TM3
			TM4
<i>Gryllus</i>	DopEcR	180	KSVTAVLGLGPSLITITVYTYIFNMHKKLRSGVPFHDKKYATALSENLSNPSHMSVFLVLFVWSTWTFATVVKLYEYVFGVQFVGLFLFI
<i>Drosophila</i>	DopEcR	176	TSAPLAIVLGLGPSLITVNGNYIFVMKKRSGSEPHDKKYATALSENLSNPSHMSVFLVAFVWWSVPIHLLRLKLYEYVFGVQSLTILFVFI
<i>Apis</i>	DopEcR	169	VSVTLVGLGPSLITITVITVYCYIFNMHKKLRSGVPFHDKKYATALSENLSNPSHMSVFLVAFVWWSVAPTAGGRIVTVVNGP-POVFFLFLFI
<i>Tribolium</i>	DopEcR	190	KVSLSLVGLGPSLITVITTYIFNMHKKLRSGYAFHDKKYATALSENLSNPSHMSVSLVAFVWWSVTPITGVKLYEYVFGVQFVGLFLFI
			TM5
			TM6
<i>Gryllus</i>	DopEcR	275	VWLGILNSFWKMLLVA-ISPQFLRALRFLCCCRKRYKGRLOAEELIGEDDD
<i>Drosophila</i>	DopEcR	271	VWLGILNSFWKMLITMSSISPQFLRALRFLCCCRKRYKGRLOAEELIGEDDD
<i>Apis</i>	DopEcR	263	VWLGIVNSFWKAVVLGA-ISPQFLRALRFLCCCRHRRLPELLELIGEDDD
<i>Tribolium</i>	DopEcR	285	VWLGILNSFWKMSMLLA-ISPQFLRALRFLCCCRKRYKGRLOAEELIGEDDD
			TM7

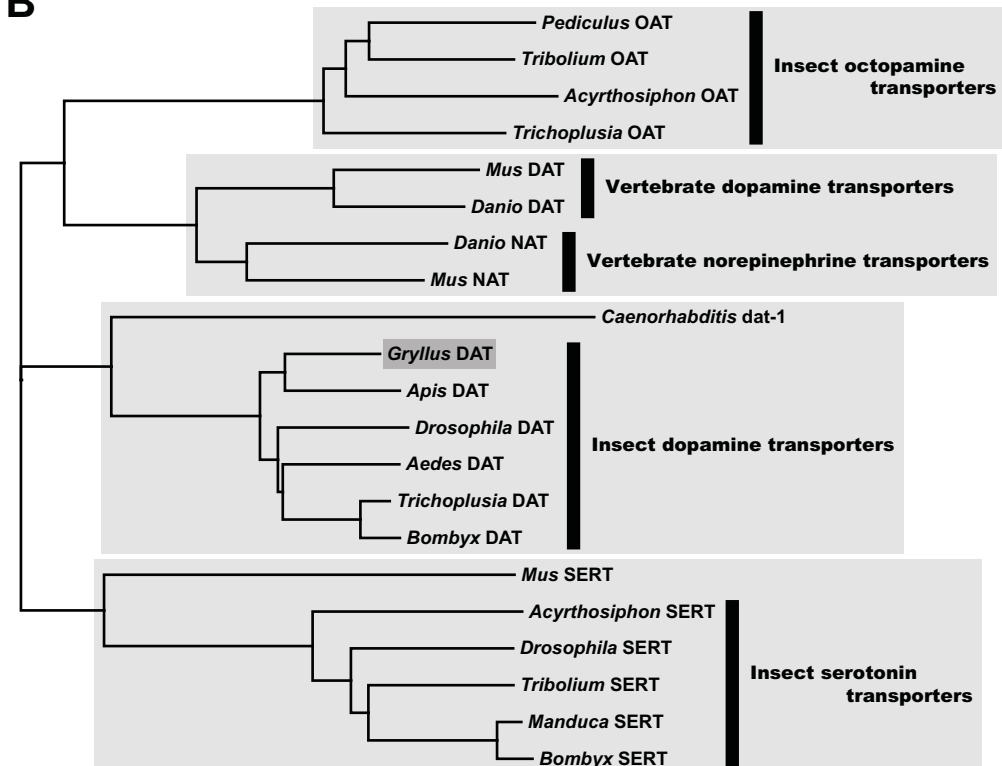
B





A

<i>Gryllus</i> DAT	1	MAP--DGAARARSPTPVGAALVRGAGGGGGGGRGGGDDVVERETWGWKVDVFLLSVIGFAVDLANVVRFPYLCYKNGGGGAPLPVYCIIMLVVGGIPLPYM		
<i>Drosophila</i> DAT	1	MSPTGHIISKST-----PTTHDNDNNISDERETWGWKVDVFLLSVIGFAVDLANVVRFPYLCYKNGGGGAPLPVYCIIMLVVGGIPLPYM		
<i>Bombyx</i> DAT	1	-----MALK--TPT-----GVVGERETWGWKVDVFLLSVIGFAVDLANVVRFPYLCYKNGGGGAPLPVYCIIMLVVGGIPLPYM		
<i>Apis</i> DAT	1	MS-----SR-----VVK-----NAPKL--NCSVDGRETWGWKVDVFLLSVIGFAVDLANVVRFPYLCYKNGGGGAPLPVYCIIMLVVGGIPLPYM		
			▲	
<i>Gryllus</i> DAT	94	ELALGGQFRKGAITCWGRIVPLFKGIGYAVVLIAFVDFYFNVIIAWALRFFASFTSSLPWTSCKGNHWNTPQCRDLVD-----	TM1	TM2
<i>Drosophila</i> DAT	84	ELALGGQFRKGAITCWGRIVPLFKGIGYAVVLIAFVDFYFNVIIAWALRFFASFTNSLPWTSCKNINWNTNCRPFESQNSARVPVIGNISDQ		
<i>Bombyx</i> DAT	71	ELALGGQFRKGAITCWGRIVPLFKGIGYAVVLIAFVDFYFNVIIAWALRFFASFTMLPWTNCDNWNTPACRPFES-----I		
<i>Apis</i> DAT	76	ELALGGQFRKGAITCWGRIVPLFKGIGYAVVLIAFVDFYFNVIIAWALRFFASFTSLPWTNCDNWNTPACRPFES-----I		
			▲	
<i>Gryllus</i> DAT	175	---GCTVSAAMADRAEDNGSH-----VTSAASEYFNRAILELHESAGLHDLGIVKWDMALCLLAVYLCYFSLWKGISTSGKVVVMTA	TM3	
<i>Drosophila</i> DAT	178	AMCNQSLIYNEFYMGSSIT--DTSVGHVEGFQSAASEYFNRAILELHESAGLHDLGIVKWDMALCLLAVYLCYFSLWKGISTSGKVVVMTA		
<i>Bombyx</i> DAT	151	NDV--NRTRIRNTDSI--SLGIAPATP-----VTSAASEYFNRAILELQCEGLHDLGIVKWDMALCLLAVYLCYFSLWKGISTSGKVVVMTA		
<i>Apis</i> DAT	169	TP--GNSSIVVEDRIDRN--SNQGYN--ST-----WYTSAAQCEYFNRAILELHESAGLHDLGIVKWDMALCLLAVYLCYFSLWKGISTSGKVVVMTA		AA
				▲
<i>Gryllus</i> DAT	256	LFPIYVLLILLVRCVTLPGSAGITRYILSPNFSAISRAEVVWVDAATQVFFSLGPGFGVLLAYASYNKYHNNVYKDALLTSVINSATSFVAGFVIF	TM4	
<i>Drosophila</i> DAT	270	LFPIYVLLILLVRCVTLPGSAGITRYILSPNFSAISRAEVVWVDAATQVFFSLGPGFGVLLAYASYNKYHNNVYKDALLTSVINSATSFVAGFVIF		
<i>Bombyx</i> DAT	236	LFPIYVLLILLVRCVTLPGSAGITRYILSPNFSAISRAEVVWVDAATQVFFSLGPGFGVLLAYASYNKYHNNVYKDALLTSVINSATSFVAGFVIF		
<i>Apis</i> DAT	255	LFPIYVLLILLVRCVTLPGSAGITRYILSPNFSAISRAEVVWVDAATQVFFSLGPGFGVLLAYASYNKYHNNVYKDALLTSVINSATSFVAGFVIF		
			TM5	TM6
<i>Gryllus</i> DAT	351	SVLGYNMAHSG-KPIQEVATEGPGLVFVYVPAAIATMPGSIFFWALIFFMMLLTGLDSSFGGSEAITALSDEFP--IICNNREIFVACLFSLYFL		TM7
<i>Drosophila</i> DAT	365	SVLGYNMAHSG-KPIQEVATEGPGLVFVYVPAAIATMPGSIFFWALIFFMMLLTGLDSSFGGSEAITALSDEFP--IICNNREIFVACLFSLYFL		
<i>Bombyx</i> DAT	331	SVLGYNMAHSG-RDVQDVATEGPGLVFVYVPAAIATMPGSIFFWALIFFMMLLTGLDSSFGGSEAITALSDEFP--IICNNREIFVACLFSLYFL		
<i>Apis</i> DAT	350	SVLGYNMAHSG-KSIQDVATEGPGLVFVYVPAAIATMPGSIFFWALIFFMMLLTGLDSSFGGSEAITALSDEFP--IICNNREIFVACLFSLYFL		
			TM8	TM9
<i>Gryllus</i> DAT	444	VGLASCTGGGYFFHLLDRIAAGYSMLFAVLFEAIAVSWIYGRFCADIIDMIGFSPGIVYRWVCWRFVAPFIFLFIIVYGLIGYEPLTYEDVYV		
<i>Drosophila</i> DAT	458	VGLASCTGGGYFFHLLDRIAAGYSMLFAVLFEAIAVSWIYGRFCADIIDMIGFSPGIVYRWVCWRFVAPFIFLFIIVYGLIGYEPLTYEDVYV		
<i>Bombyx</i> DAT	424	VGLASCTGGGYFFHLLDRIAAGYSMLFAVLFEAIAVSWIYGRFCADIIDMIGFSPGIVYRWVCWRFVAPFIFLFIIVYGLIGYEPLTYEDVYV		
<i>Apis</i> DAT	443	VGLASCTGGGYFFHLLDRIAAGYSMLFAVLFEAIAVSWIYGRFCADIIDMIGFSPGIVYRWVCWRFVAPFIFLFIIVYGLIGYEPLTYEDVYV		
			TM10	TM11
<i>Gryllus</i> DAT	539	PGWANLIGWLIAGSSIVMIPGMAYKLLTTPGCFQRLKYLTTTPWRDQ--IFA--INGVQ--TDSQHRLA--SSPQA--DDV-----		
<i>Drosophila</i> DAT	553	PGWANLIGWLIAGSSIVMIPGMAYKLLTTPGCFQRLKYLTTTPWRDQ--QSSMAMVINGVT--TEVVVRL--TDTEFAKSPVDV-----		
<i>Bombyx</i> DAT	519	PGWANLIGWLIAGSSIVMIPGMAYKLLTTPGCFQRLKYLTTTPWRDQ--NQTVHNCMVSSGGGVRLMSAVQ--PTTPQPPGVANIPAAAPAL--		
<i>Apis</i> DAT	538	PGWANLIGWLIAGSSIVMIPGMAYKLLTTPGCFQRLKYLTTTPWRDQ--QSSMAMVINGVT--TEVVVRL--TDTEFAKSPVDV-----		
			TM12	
<i>Gryllus</i> DAT		-----		
<i>Drosophila</i> DAT		-----		
<i>Bombyx</i> DAT	613	ASSPALV-----		
<i>Apis</i> DAT	626	VMIQSRNVNGDPPPEPV		

B

Ef1alpha

DAT

RT(+)

RT(-)

Central brain
SOG

Lamina + Medulla + Retina
CC + CA

Thoracic muscles
Salivary gland
Midgut

Malpighian tubules
Testicle
Ovary

