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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*

INTRODUCTION

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

In insects, dopamine functions as a neurotransmitter/neurohormone that is synthesized in and released from dopaminergic neurons in the nervous system, and also serves as a precursor of melanin that plays important roles in cuticular sclerotization/pigmentation and in the innate immune system. Dopamine biosynthesis 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 D_1-like dopamine receptors (Dop1), invertebrate dopamine receptors (INDRs, also known as Dop2), the D_2-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 (IP_3) 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 D_2-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.

MATERIALS AND METHODS

Animals

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

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

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

Next, to obtain the 5’ and 3’ region of target mRNAs, we performed 5’ and 3’ rapid 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/epestfind). 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* D_2-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 algorism 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 algorism 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 ºC 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 (Arg^{29} and Arg^{30}), 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

D_1-like and invertebrate dopamine receptors: We identified the D_1-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 D_1-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).

D_2-like dopamine receptor: We identified an insect D_2-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 D_2-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 D_2-like dopamine receptors also indicates that *Gryllus Dop3* is closely related to insect D_2-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-Grelin 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 (Ser^{32}, Ser^{50}, Tyr^{65} and Ser^{78}) which are conserved in *Drosophila* TH (pale, see Figure 1). The Ser^{32} is conserved in TH proteins of both vertebrates and invertebrates, and enzymatic activity of *Drosophila* TH is increased by phosphorylation at Ser^{32} *in vitro* (Vié et al., 1999). The Ser^{40} residue of vertebrate TH proteins, which is homologous to the Ser^{32} 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 D_1-like receptors, INDRs and D_2-like receptors.

Recently another GPCR-type receptor, DopEcR, was identified in *D. melanogaster*, which shows high affinity for dopamine as well as ecdysteroids (Srivastava et al., 2005). D_1-like receptors are coupled to $G_{\alpha s}$ protein, which subsequently activates adenylyl cyclase to produce cAMP, and the INDRs are coupled to Ca^{2+} signaling as well as cAMP (Beggs et al., 2011). On the other hand, D_2-like dopamine receptors are coupled to $G_{\alpha i}$ protein that inhibits adenylyl cyclase. D_2-like dopamine receptors can also activate the mitogen-activated protein kinase (MAPK) pathway (Yan et al., 1999). Activation of DopEcR by dopamine leads to an increase in intracellular cAMP levels as well as activation of the phosphoinositol 3-phosphate pathway. Ecdysteroids, in contrast, inhibit the effects of dopamine and induce the activation of the MAPK pathway (Srivastava et al., 2005). In the present study, we searched GPCRs structurally associated with *Gryllus* DopEcR in order to construct a molecular phylogenetic tree of DopEcR, and found that the vertebrate GPR21 and GPR52 genes are most similar to insect DopEcR. GPR21 and GPR52 were first identified from the human expressed sequence tags database and genome database (O'Dowd et al., 1997; Sawzdargo et al., 1999), and their homologues were then found in other vertebrate genomes (see Table 2). Although the natural agonists, downstream signal transduction pathways and physiological roles of the vertebrate GPR21 and GPR52 are still unknown, the mRNA of human GPR21 is expressed in several brain areas (e.g. the frontal cortex, caudate nucleus, putamen, thalamus) (O'Dowd et al., 1997). Gene function analyses of the DopEcR/GPR21/GPR52 family are necessary to reveal the physiological roles of the novel GPCR family.

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

Central nervous system: Hamada et al. (2009) cloned partial cDNA fragments of the *Gryllus Dop1* and *Dop2* genes, and examined their mRNA distributions in the brain. They reported that mRNAs of both dopamine receptors were expressed predominantly in the Kenyon cells of the mushroom bodies in the cricket brain. In *Drosophila*, the DAMB (a homologue of *Gryllus* Dop2) is exclusively expressed in the mushroom bodies (Han et al., 1996), in which the dopaminergic system plays essential roles in learning and memory (Waddell, 2010). In the brain of the honeybee and silkmoth, mRNAs of dopamine receptors are also detected in the Kenyon cells of the mushroom bodies (Kurshan et al., 2003; Kyle et al., 2005; Mitsumasu et al., 2008). In the cricket, pharmacological inhibition of D_1-like dopamine receptor impaired aversive olfactory and visual conditioning and memory recall (Unoki et al., 2005; Unoki et al., 2006; Mizunami et al., 2009). In *Drosophila*, the D_1-like dopamine receptor DopR (a homologue of *Gryllus* Dop1) regulates caffeine-induced arousal in the mushroom bodies (Andretic et al., 2008), as well as stress-induced arousal in the central complex and sleep-wake arousal in the lateral-ventral neurons (Lebestky et al., 2009). Moreover, the *DopEcR* gene is strongly expressed in the cricket CNS although its physiological functions in the nervous system are still unknown in insects. Contrary to the *Gryllus Dop1*, *Dop2* and *DopEcR* genes, which are expressed in all neuronal tissues examined in this study, the *Gryllus* D_2-like dopamine receptor gene, *Dop3*, showed restricted expression in the brain and SOG, and its expression was not detected in the optic lobes and CC + CA. Draper et al. (2007) examined the distribution of the *Drosophila* D_2-like receptor (DD2R) in the CNS using anti-DD2R antibodies, and found that a small number of neurons including the Ap-let cohort of peptidergic neurons was immunoreactive in the larval and adult CNS. They also generated *DD2R* RNA-interference (RNAi) lines and demonstrated that the RNAi-mediated knock-down of the *DD2R* gene resulted in the reduction of locomotor activity in the adult flies. On the other hand, in the honeybee, RNAi-mediated knockdown of

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

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

Salivary gland: In insects, the aminergic and peptidergic systems control salivation (Ali, 1997; Walz et al. 2006). In *G. bimaculatus*, dopaminergic innervations in the salivary gland originate from the SOG (SN1 neurons) (Helle et al., 1995; Hörner et al., 1995; Ali, 1997). In the cockroach salivary gland, dopamine induces production of cAMP that acts as a second messenger in the acinar cells to cause the secretory response (Grewe and Kebabian, 1982; Gray et al., 1984; Marg et al. 2004; Rietdorf et al. 2005). Our study demonstrates that 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 hymenopretans 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 D_2-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

D_2-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 D_2-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 D_1-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.

In summary, we identified six genes involved in the biosynthesis, transduction and re-uptake of dopamine in the cricket *G. bimaculatus*. Two TH isoforms showed isoform-specific distribution in cricket tissues. Tissue-specific expression analysis of dopamine receptor genes showed that the dopaminergic system is widely distributed in the cricket, and that the dopaminergic system might regulate various aspects of physiological phenomena via distinct 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 (BH_4)-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* D_1-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 D_2-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 proetins are as follows: *Drosophila* Dop1 (*Drosophila melanogaster*, AAA85716), *Apis* Dop1 (*Apis mellifera*, NP_001011595), *Nasonia* Dop1 (*Nasonia vitripennis*, XP_001606438), *Acyrthosiphon* Dop1 (*Acyrthosiphon 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* D_1-like (*Aplysia californica*, NP_001191631), *Danio* D_1 (*Danio rerio*, NP_001129448), *Danio* D_5-like 1 (*Danio rerio*, XP_001341592), *Danio* D_5-like 2 (*Danio rerio*, XP_692025), *Mus* D_1 (*Mus musculus*, NP_034206), *Mus* D_2 (*Mus musculus*, NP_034207), *Mus* D_3 (*Mus musculus*, NP_031903), *Mus* D_4 (*Mus musculus*, NP_031904), *Mus* D_5 (*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* D_2-like dopamine receptor (Dop3) with other D_2-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 D_1-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* D_1 (*Mus musculus*, NP_034206), *Mus* D_2 (*Mus musculus*, NM_010077), *Mus* D_3 (*Mus musculus*, NM_007877), *Mus* D_4 (*Mus musculus*, NM_007878), *Mus* D_5 (*Mus musculus*, NP_038531), *Danio* D_{2a} (*Danio rerio*, NM_183068), *Danio* D_{2b} (*Danio rerio*, NM_197936), *Danio* D_2*-like* (*Danio rerio*, NM_197935), *Danio* D_3 (*Danio rerio*, NM_183067), *Danio* D_{4a} (*Danio rerio*, NM_001012616), *Danio* D_{4b} (*Danio rerio*, NM_001012618), *Danio* D_4*-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 H_2 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 proetins 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* OAβ_1 (*Drosophila melanogaster*, AJ880687), *Drosophila* OAβ_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 H_2 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 resides 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), *Acyrthosiphon* OAT (*Acyrthosiphon pisum*, XP_001949303), *Drosophila* SERT (*Drosophila melanogaster*, NP_523846), *Bombyx* SERT (*Bombyx mori*, NP_001037436), *Manduca* SERT (*Manduca sexta*, AAN59781), *Tribolium* SERT (*Tribolium*

castaneum, XP_968717), *Acyrthosiphon* SERT (*Acyrthosiphon pisum*, XP_001944311), *Mus* SERT (*Mus musculus*, NP_034614).

Figure 10

Tissue-specific expression pattern of *Gryllus DAT* gene. The cDNA fragments of *Gryllus DAT* gene 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.

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

Degenerate primers		
	Forward primer	Reverse primer
TH (C-terminal region)	5’-CACYTCGAANGGYCGSGACAT-3’	
DopEcR	5'-GGTNTAYACNTTYATGTGGAT-3'	5'- GRCCATCCARCAYTGRCA -3'
DAT	5'-GGCATHCCYYTNTTCTACATGG-3'	5'-ATDATGACGTTRTAGTAGAARTCNACGTA-3'

Primers for full-length/partial ORF amplification		
	Forward primer	Reverse primer
TH	5’-CCGCGACAACAACTCTCAG-3’	5'-CATGCCGGTGGACACCCAC-3'
Dop1	5’-GCGCCGCCATGGAGGACGACG-3’	5’-GCGGCCGTCTCTTCAGATGG-3’
Dop2	5’-GCCGACGAAACAACTCGAG-3’	5’-CCGGCTGTTTGTCAGGTGG-3’

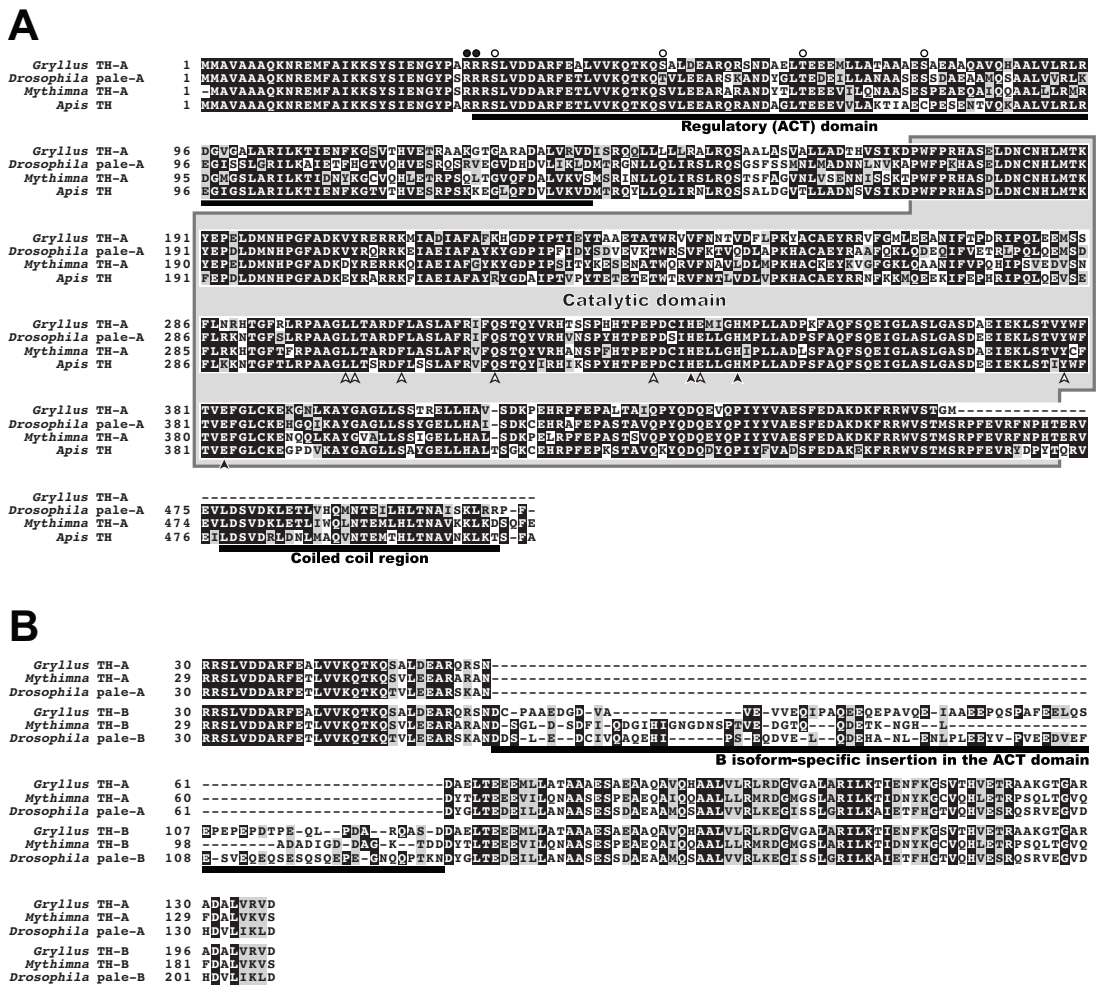
Dop3	5’-GCCCTTCTTCACGTGCAAC-3’	5’-CCAGCGCGTGTTGTTACG-3’
DopEcR	5’-CCCTGCACCCCTCACCGCGACTATG-3’	5’-CCAGCGCGTGTTGTTACG-3’
DAT	5’-CGCTGAGGAGCTGCAGATG-3’	5’-GAGCACCTTTTGGCAAATAGAGAAATCACAC-3’

Primers for tissue-specific expression analysis

	Forward primer	Reverse primer
TH common region	5’-GGCCTTCCGGATCTTCC-3’	5’-GGGTGTCCACCGGCATG-3’
TH-A isoform	5'-CCGCGACAACAACTCTCAG-3'	5'-CCGTCAGCTCAGCGTCGTTG-3'
TH-B isoform	5'-CCGCGACAACAACTCTCAG-3'	5'-CCGTCAGCTCAGCGTCATC-3'
Dop1	5’-CGCCGTACCACGTGTCTG-3’	5’-CGCCGAGTTGGAGTAGC-3’
Dop2	5’-CCAACTCCAGCATGAATCC-3’	5’-GAGTGCGCCAGCATCATGG-3’

Dop3	5’-GCCATGTGCACCAAGCTG-3’	5’-CCAGCGCGTGTTGTTACG-3’
DopEcR	5’-CGTACAGCGGTGGGTGTATGG-3’	5’-CGTCTGCACCGTCTCGTAGC-3’
DAT	5’-GTGGCGAGATCATCAAATAGCTGC-3’	5’-GAGCACCTTTTGGCAAATAGAGAAATCACAC-3’
Ef1alpha	5’-GTGTTCTGAAGCCAGGTATGG-3’	5’-CTCCAGCAACATAACCACGAC-3’

1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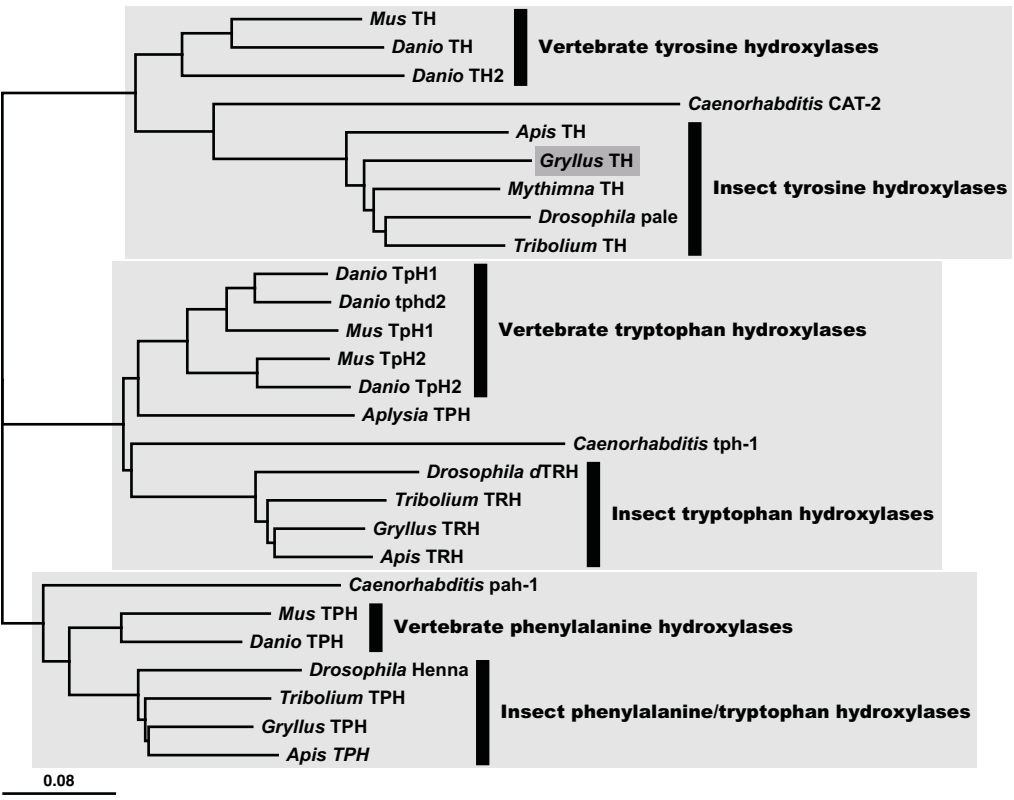
Drosophila pale-A
Mythimna TH-A
Apis TH
Regulatory (ACT) domain
Catalytic domain
Coiled coil region
B
B isoform-specific insertion in the ACT domain


Mus TH
Danio TH
Danio TH2
Vertebrate tyrosine hydroxylases
Caenorhabditis CAT-2
Apis TH
Gryllus TH
Mythimna TH
Drosophila pale
Tribolium TH
Insect tyrosine hydroxylases
Danio TpH1
Danio tphd2
Mus TpH1
Mus TpH2
Danio TpH2
Vertebrate tryptophan hydroxylases
Aplysia TPH
Caenorhabditis tph-1
Drosophila dTRH
Tribolium TRH
Gryllus TRH
Apis TRH
Insect tryptophan hydroxylases
Caenorhabditis pah-1
Mus TPH
Danio TPH
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Drosophila Henna
Tribolium TPH
Gryllus TPH
Apis TPH
Insect phenylalanine/tryptophan hydroxylases
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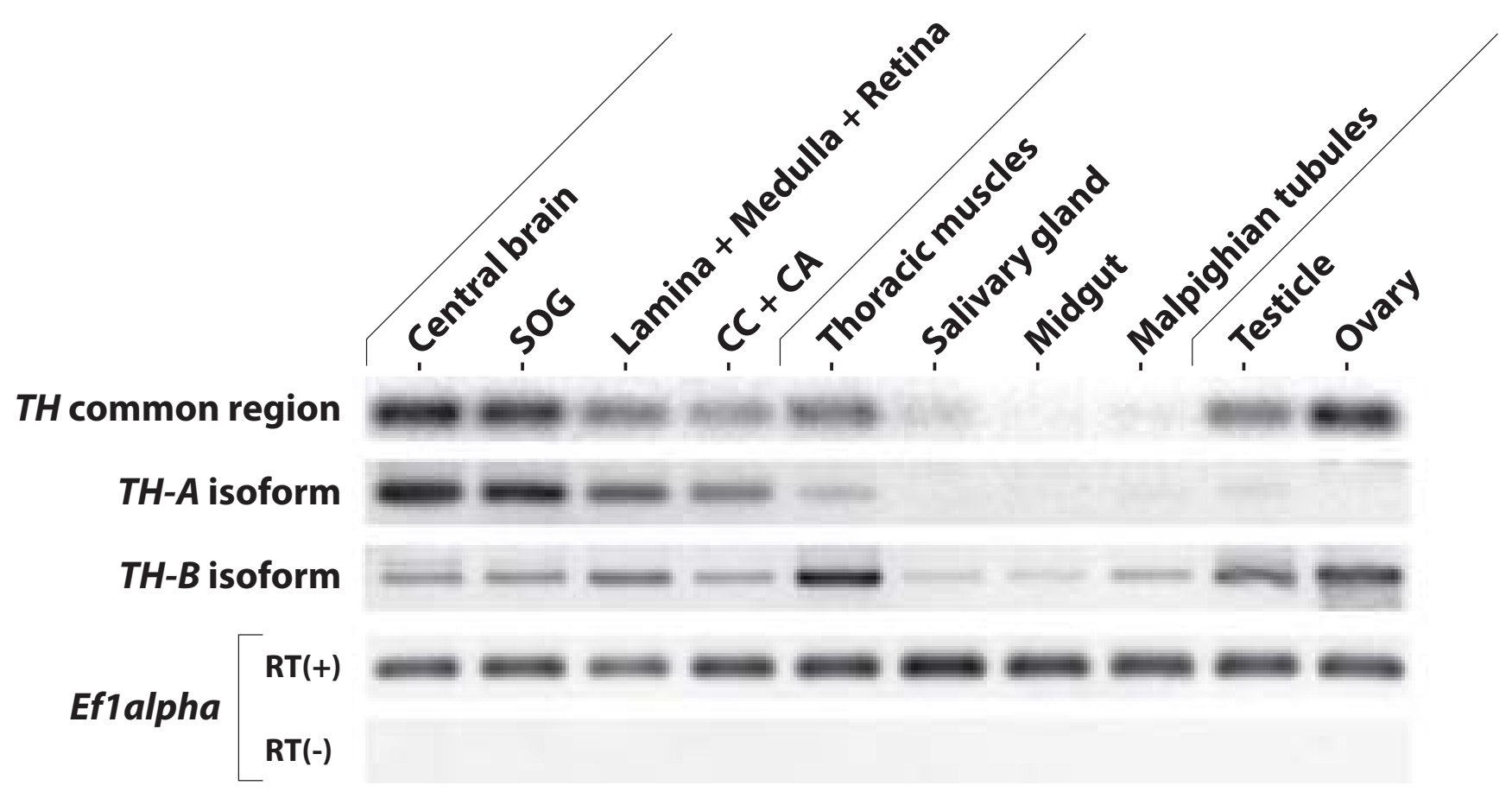
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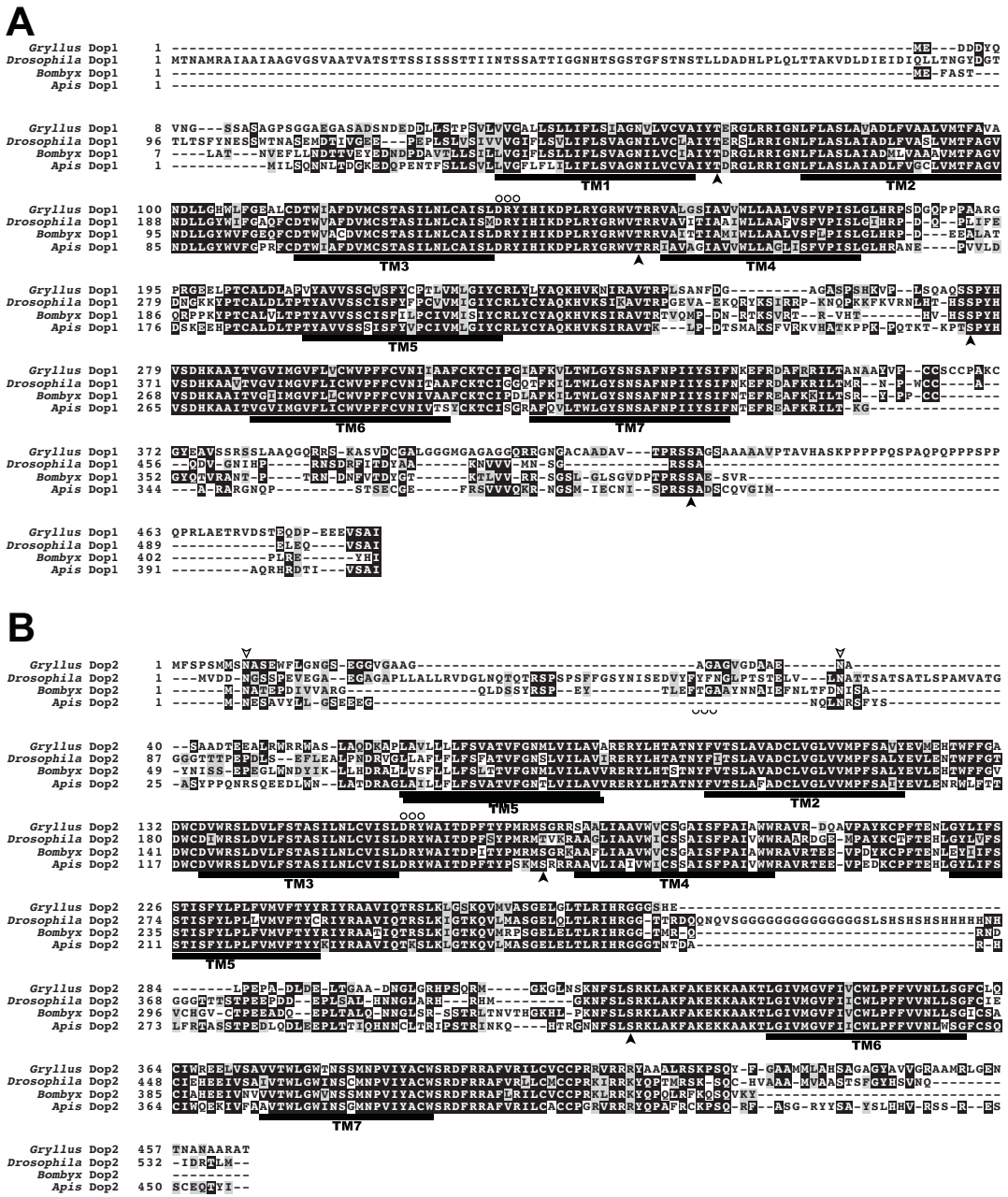
SOG
Lamina + Medulla + Retina
CC + CA
Thoracic muscles
Salivary gland
Midgut
Malpighian tubules
Testicle
Ovary
TH common region
TH-A isoform
TH-B isoform
Ef1alpha
RT(+)
RT(-)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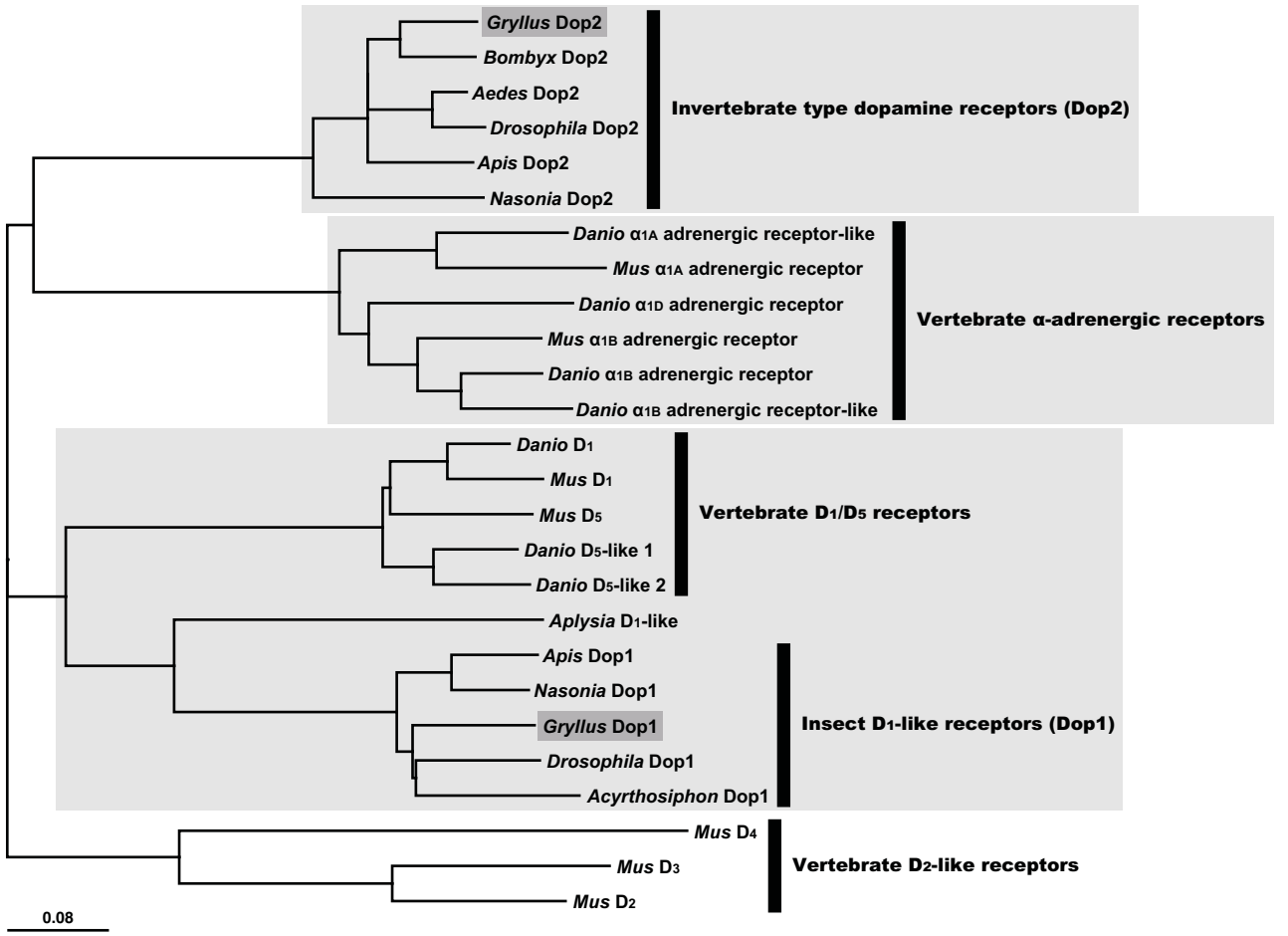
Drosophila Dop1
Bombyx Dop1
Apis Dop1
TM1
TM2
TM3
TM4
TM5
TM6
TM7
B
Gryllus Dop2
Drosophila Dop2
Bombyx Dop2
Apis Dop2
TM1
TM2
TM3
TM4
TM5
TM6
TM7


Gryllus Dop2
Bombyx Dop2
Aedes Dop2
Drosophila Dop2
Apis Dop2
Nasonia Dop2
Invertebrate type dopamine receptors (Dop2)
Danio α1A adrenergic receptor-like
Mus α1A adrenergic receptor
Danio α1D adrenergic receptor
Mus α1B adrenergic receptor
Danio α1B adrenergic receptor
Danio α1B adrenergic receptor-like
Vertebrate α-adrenergic receptors
Danio D1
Mus D1
Mus D5
Danio D5-like 1
Danio D5-like 2
Vertebrate D1/D5 receptors
Aplysia D1-like
Apis Dop1
Nasonia Dop1
Gryllus Dop1
Drosophila Dop1
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Mus D4
Mus D3
Mus D2
Vertebrate D2-like receptors
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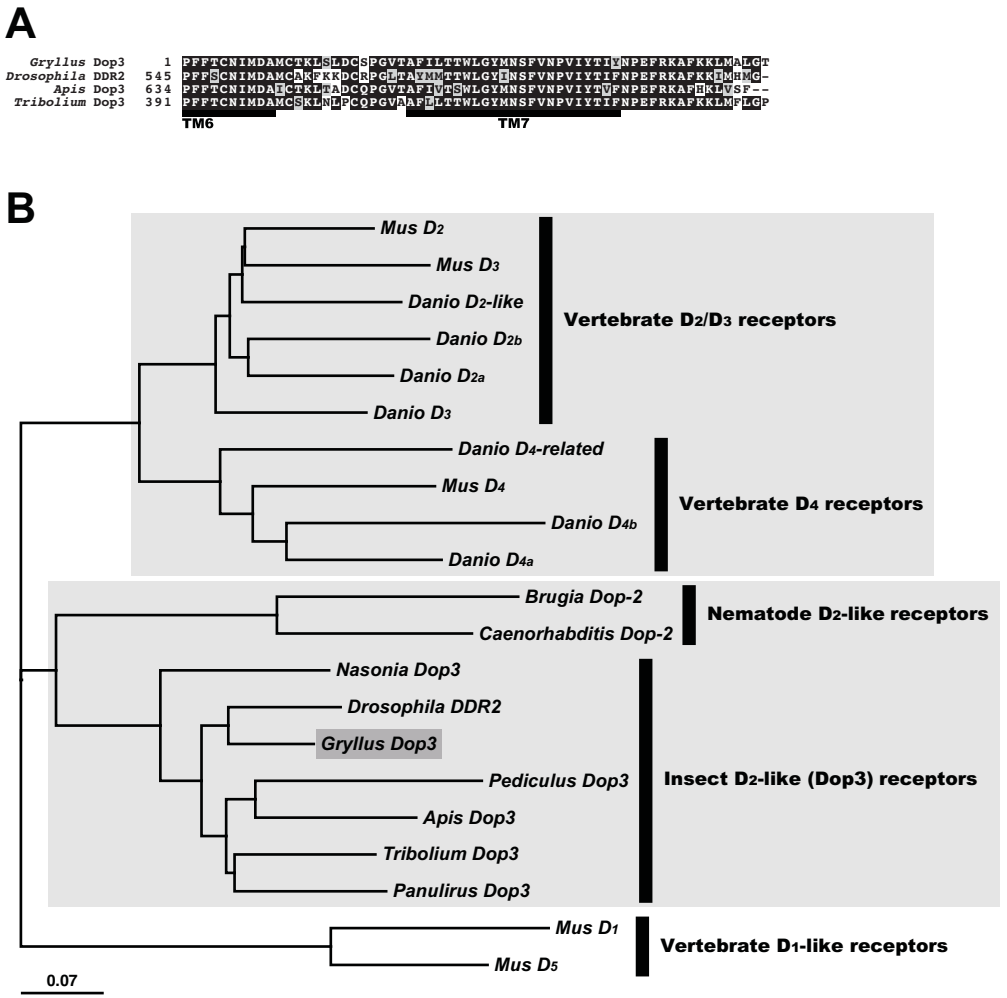
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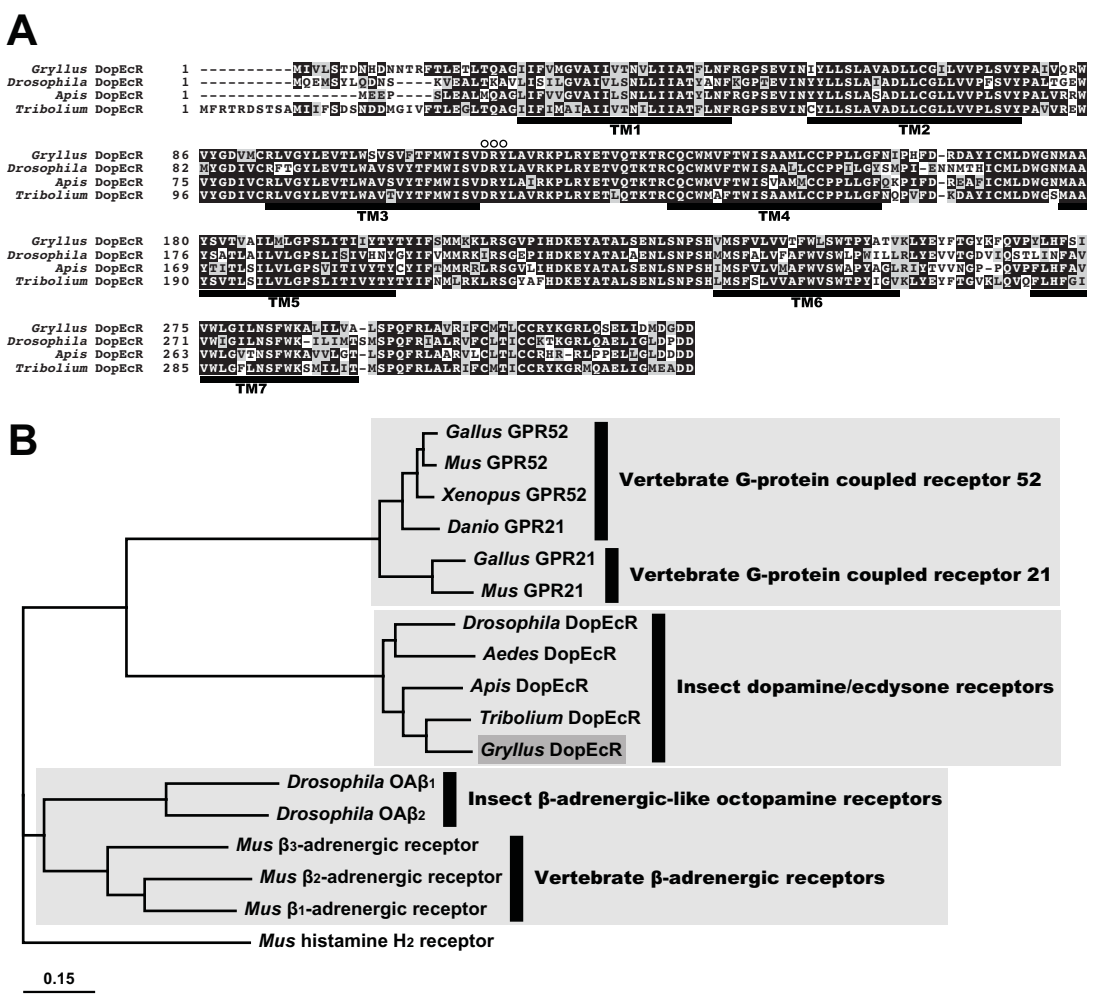
Drosophila DDR2 545 PFFSCNIMDAMCAKFKKDCKPGLTAYMMTTWLGYFNSFVNPVIYTIFNPEFRKAFKKIMHMG-
Apis Dop3 634 PFFTCNIMDAHCTKLTADCQPGVTAFIVTSWLGYMNSFVNPVIYTVFNPEFRKAFHKLVSF--
Tribolium Dop3 391 PFFTCNIMDAMCSKLNLPCQPGVAAFLLTTWLGYMNSFVNPVIYTIFNPEFRKAFKKLMFLGP
TM6
TM7
B
Mus D2
Mus D3
Danio D2-like
Danio D2b
Danio D2a
Danio D3
Vertebrate D2/D3 receptors
Danio D4-related
Mus D4
Danio D4b
Danio D4a
Vertebrate D4 receptors
Brugia Dop-2
Caenorhabditis Dop-2
Nematode D2-like receptors
Nasonia Dop3
Drosophila DDR2
Gryllus Dop3
Pediculus Dop3
Apis Dop3
Tribolium Dop3
Panulirus Dop3
Insect D2-like (Dop3) receptors
Mus D1
Mus D5
Vertebrate D1-like receptors
0.07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Drosophila DopEcR
Apis DopEcR
Tribolium DopEcR
TM1
TM2
TM3
TM4
TM5
TM6
TM7
B
Gallus GPR52
Mus GPR52
Xenopus GPR52
Danio GPR21
Vertebrate G-protein coupled receptor 52
Gallus GPR21
Mus GPR21
Vertebrate G-protein coupled receptor 21
Drosophila DopEcR
Aedes DopEcR
Apis DopEcR
Tribolium DopEcR
Gryllus DopEcR
Insect dopamine/ecdysone receptors
Drosophila OAβ1
Drosophila OAβ2
Insect β-adrenergic-like octopamine receptors
Mus β3-adrenergic receptor
Mus β2-adrenergic receptor
Mus β1-adrenergic receptor
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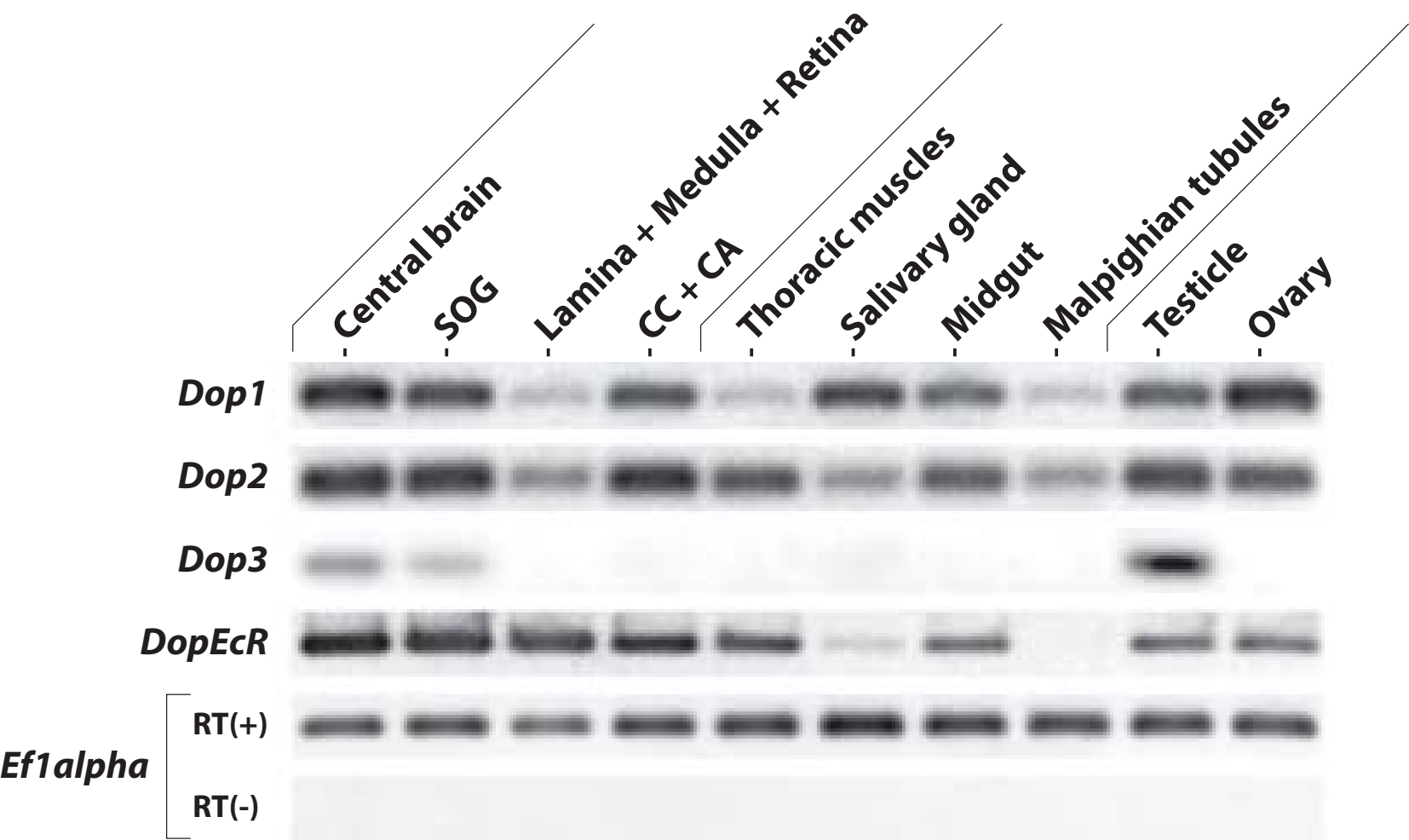
Central brain
SOG
Lamina + Medulla + Retina
CC + CA
Thoracic muscles
Salivary gland
Midgut
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Testicle
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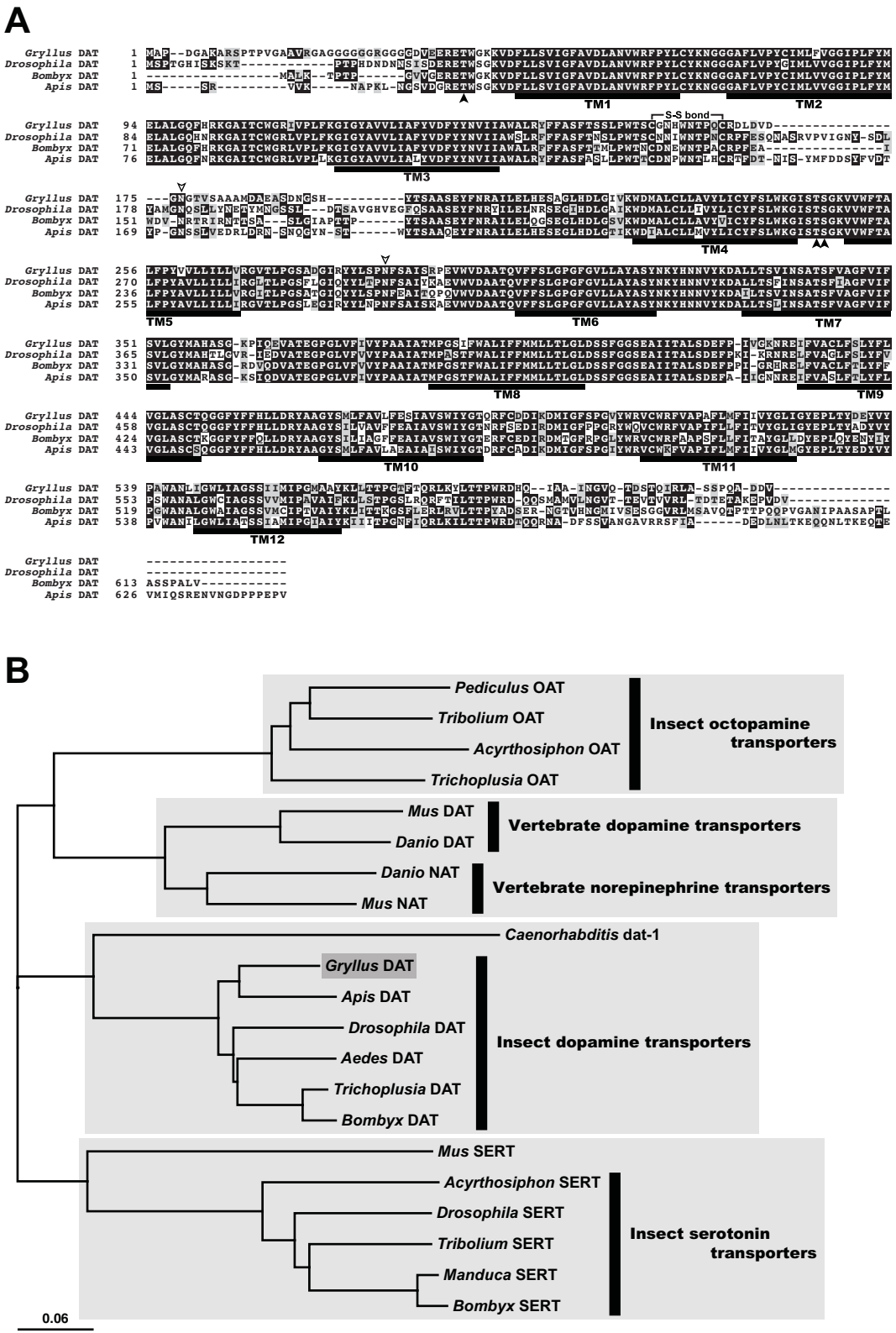
DopEcR
RT(+)
RT(-)
Ef1alpha


A
Gryllus DAT
Drosophila DAT
Bombyx DAT
Apis DAT
TM1
TM2
S-S bond
TM3
TM4
TM5
TM6
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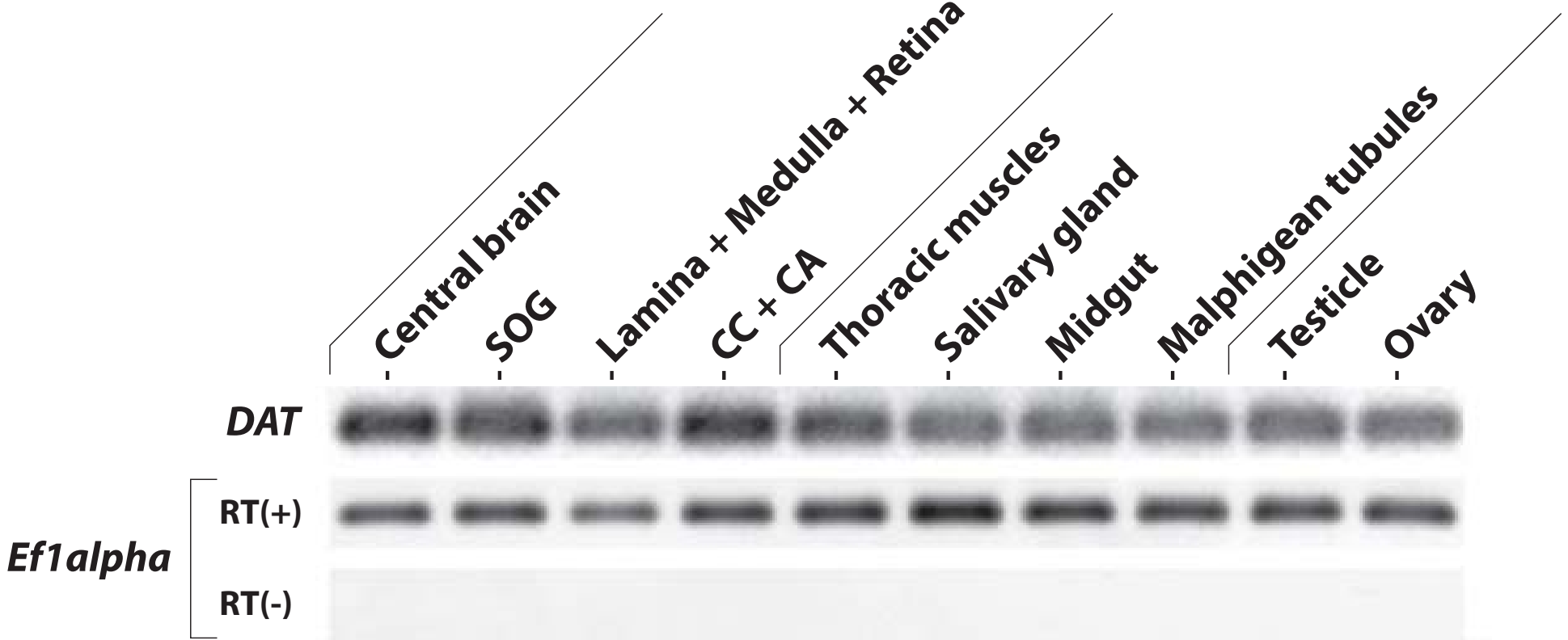
TM8
TM9
TM10
TM11
TM12
B
Pediculus OAT
Tribolium OAT
Acyrthosiphon OAT
Trichoplusia OAT
Insect octopamine transporters
Mus DAT
Danio DAT
Vertebrate dopamine transporters
Danio NAT
Mus NAT
Vertebrate norepinephrine transporters
Caenorhabditis dat-1
Gryllus DAT
Apis DAT
Drosophila DAT
Aedes DAT
Trichoplusia DAT
Bombyx DAT
Insect dopamine transporters
Mus SERT
Acyrthosiphon SERT
Drosophila SERT
Tribolium SERT
Manduca SERT
Bombyx SERT
Insect serotonin transporters
0.06


Central brain
SOG
Lamina + Medulla + Retina
CC + CA
Thoracic muscles
Salivary gland
Midgut
Malphigean tubules
Testicle
Ovary
DAT
Ef1alpha
RT(+)
RT(-)