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Identification and expression analysis of the genes involved in serotonin biosynthesis and transduction in the field cricket *Gryllus bimaculatus*

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Running title: 5-HT-related genes in the cricket

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ABSTRACT

Serotonin (5-HT) modulates various aspects of behaviors such as aggressive behavior and circadian behavior in the cricket. To elucidate the molecular basis of the cricket 5-HT system, we identified 5-HT-related genes in the field cricket *Gryllus bimaculatus* DeGeer. Complementary DNA of tryptophan hydroxylase (TRH) and phenylalanine-tryptophan hydroxylase (TPH) which convert tryptophan into 5-hydroxy-L-tryptophan (5-HTP), and that of aromatic L-amino acid decarboxylase (AADC) which converts 5-HTP into 5-HT were isolated from a cricket brain cDNA library. In addition, four 5-HT receptor genes (5-HT_{1A} , 5-HT_{1B} , 5-HT_{2a} , and 5-HT_7) were identified. Expression analysis of *TRH* and *TPH*, which are selectively involved in neuronal and peripheral 5-HT synthesis in *Drosophila*, suggested that two 5-HT synthesis pathways co-exist in the cricket neuronal tissues. The four of 5-HT receptor genes were expressed in various tissues at differential expression levels, suggesting that the 5-HT system is widely distributed in the cricket.

Keywords: serotonin, tryptophan hydroxylase, phenylalanine-tryptophan hydroxylase, aromatic L-amino acid decarboxylase, serotonin receptors, *Gryllus bimaculatus*.

INTRODUCTION

Crickets show various behaviors regulated by biogenic amines. Pharmacological and behavioral studies have revealed the involvement of specific biogenic amines in modulating various aspects of behavior. For example, the octopaminergic and dopaminergic systems respectively mediate reward and punishment signals in olfactory and visual learning (Unoki et al., 2005; Unoki et al., 2006). In the optic lobes, serotonergic neurons regulate the circadian pacemakers to set the day state (Saifullah and Tomioka, 2002). Pharmacological inhibition of aminergic neurotransmitter biosynthesis revealed that depletion of serotonin (5-HT) and dopamine/octopamine had differential effects on aggressive and escape behaviors of male crickets (Stevenson et al., 2000). Furthermore, hormonally released octopamine and 5-HT reciprocally control the length of the mating interval in male crickets (Nagao et al., 1991). Although physiological and behavioral aspects of the aminergic system have been extensively studied, the molecular basis of the cricket aminergic system has not been investigated yet. In the present study, we investigated the molecular basis of the 5-HT system in the cricket *Gryllus bimaculatus* DeGeer.

The biogenic monoamine 5-HT is an ancient intracellular signaling molecule found in all phyla that possess nervous systems. In the nervous system of both vertebrates and invertebrates, 5-HT functions as a neurotransmitter/modulator, or as a neurohormone that modulates various principal behaviors, such as feeding, circadian behavior, sleep, sexual behavior, and social behavior.

5-HT synthesis requires two enzymes, tryptophan hydroxylase and aromatic L-amino acid decarboxylase (AADC) (Figure 1). As first step of 5-HT synthesis, tryptophan

hydroxylase converts L-tryptophan into 5-hydroxy-L-tryptophan (5-HTP). Then, AADC converts 5-HTP into 5-HT. In the fruit fly *Drosophila melanogaster*, tryptophan hydroxylase is encoded by two genes, *dTPH* and *dTRH* (Coleman and Neckameyer, 2005). Both gene products have tryptophan hydroxylase activity *in vivo*, but the *dTPH* gene is expressed in non-neuronal tissues, and the *dTRH* gene is expressed in the neural tissues (Neckameyer et al., 2007). Homologues of these genes were found in the genome of other insects such as bees, beetles, and moths, but their tissue-specific expression patterns have not been examined. AADC is not only involved in the biosynthesis of serotonin but also dopamine, and thus, AADC is expressed in serotonergic and dopaminergic neurons in the central nervous system (CNS).

All of the known insect 5-HT receptors belong to the seven-transmembrane segments (7TM)-containing G protein-coupled receptor (GPCR) superfamily. In *Drosophila*, five 5-HT receptors (5-HT_{1A}, 5-HT_{1B}, 5-HT_{2α}, 5-HT_{2β}, and 5-HT₇) were identified (Brody and Cravchik, 2000), and their homologues were found in some other insects. The 5-HT_{2β} receptor has not yet been characterized in any insect (Blenau and Thamm, 2011). The other four 5-HT receptor subtypes are known to be coupled with subtype-specific signal transduction mechanisms (Tierney, 2001). Behavioral pharmacology and genetic studies on the insect 5-HT receptors revealed the specific involvement of the receptor subtypes in modulating behaviors such as circadian behavior and aggressive behavior (Yuan et al., 2005; Yuan et al., 2006; Nichols 2007; Johnson et al., 2009).

To elucidate the molecular basis of the cricket 5-HT system, we identified seven genes involved in synthesis and transduction of 5-HT expressed in the brain of the cricket, *G. bimaculatus*. We obtained the full-length open reading frame (ORF) clones of the *TPH*, *TRH*, and *AADC* genes, as well as the partial ORF clones of four 5-HT receptor genes (5-HT_{1A}, 5-HT_{1B},

5-HT_{2a}, and *5-HT₇*). Expression analysis of the 5-HT-related genes revealed that the 5-HT system is widespread in the CNS and in peripheral tissues of the cricket.

RESULTS

Nucleotide Sequence and Structural Features of *Gryllus TRH* cDNA

We identified a tryptophan hydroxylase gene, *TRH*, expressed in the cricket brain. We performed RT-PCR with the gene-specific primers (GSPs) designed at the 5' and 3' UTRs of the gene, and obtained 1706-bp and 1823-bp cDNA fragments of *TRH* (GenBank accession numbers: AB618095 and AB626809, respectively). The short variant contains an ORF spanning 13–1629 bp, resulting in a protein product of 538 amino acids. The long variant contains an ORF spanning 13–1746 bp, resulting in a protein product of 577 amino acids. Several functional domains and residues, such as the N-terminal regulatory (ACT) domain, the catalytic domain, the C-terminal coiled-coil region involved in tetramerization, tetrahydrobiopterin (BH4)-binding sites, and iron binding sites, are conserved in *Gryllus* TRH (Fig. 2(A)). The long variant contains the 39 amino acid insertion in the N-terminal region of the catalytic domain (between amino acids 179 and 180 of the short variant) (supplementary figure 1).

Comparison of the deduced amino acid sequence of *Gryllus* TRH with those of other TRH, cloned from both vertebrates and invertebrates, indicated that *Gryllus* TRH is structurally most similar to the other known insect TRH (64% identical to *Drosophila* TRH, 66.5% identical to *Tribolium* TRH, and 74.9% identical to *Apis* TRH). The molecular phylogenetic analysis of aromatic amino acid hydroxylases using the full-length of the deduced amino acid sequence of the *Gryllus* TRH gene and those of various aromatic amino acid hydroxylases also indicated that *Gryllus* TRH is closely related to the insect TRH proteins (Fig. 3).

Nucleotide Sequence and Structural Features of *Gryllus TPH* cDNA

We identified a phenylalanine-tryptophan hydroxylase gene, *TPH*, expressed in the cricket brain. We performed RT-PCR with the GSPs designed at the 5' and 3' UTRs of the gene, and obtained a 1611-bp cDNA fragment of *TPH* (GenBank accession number: AB618096). This clone contains an ORF spanning 68–1435 bp, resulting in a protein product of 455 amino acids. Several functional domains and residues, such as the N-terminal regulatory (ACT) domain, the catalytic domain, the C-terminal coiled-coil region involved in BH4-binding sites, and iron binding sites, are conserved in *Gryllus* TPH (Fig. 2(B)).

Comparison of the deduced amino acid sequence of *Gryllus* TPH with those of other invertebrate TPH and vertebrate phenylalanine-4-hydroxylase indicated that *Gryllus* TPH is structurally most similar to the other known insect TPH (75.9% identical to *Apis* TPH isoform 2, 75.5% identical to *Papilio* TPH, and 71.6% identical to *Drosophila* TPH isoform A). The molecular phylogenetic analysis of aromatic amino acid hydroxylases using the full-length of the deduced amino acid sequence of the *Gryllus* *TPH* gene and those of various aromatic amino acid hydroxylases also indicated that *Gryllus* TPH is closely related to the insect TPH proteins (Fig. 3).

Nucleotide Sequence and Structural Features of *Gryllus AADC* cDNA

We identified an aromatic L-amino acid decarboxylase gene, *AADC*, expressed in the cricket brain. We performed RT-PCR with the GSPs designed at 5' and 3' UTRs of the *Gryllus AADC* gene, and obtained a 1522-bp cDNA fragment (GenBank accession number: AB618097). This clone contains an ORF spanning 12–1454 bp, resulting in a protein product of 480 amino acids. The catalytic domain and several functional residues, such as the pyridoxal 5'-phosphate binding sites and the catalytic loop, are conserved in *Gryllus* AADC (Fig. 4(A)).

Comparison of the deduced amino acid sequence of *Gryllus* AADC with those of other AADC indicated that *Gryllus* AADC is structurally most similar to the other known insect AADC (75.2% identical to *Tribolium* AADC, 75.3% identical to *Bombyx* AADC, and 74.2% identical to *Drosophila* AADC). The molecular phylogenetic analysis of AADC proteins using the full-length of the deduced amino acid sequence of the *Gryllus AADC* gene and those of various amino acid decarboxylases also indicated that *Gryllus* AADC is closely related to the insect AADC proteins (Fig. 4(B)).

Nucleotide Sequence and Structural Features of cDNAs of the *Gryllus* 5-HT receptor genes

We identified four 5-HT receptor genes (*5-HT_{1A}*, *5-HT_{1B}*, *5-HT_{2α}*, and *5-HT₇*) expressed in the cricket brain. We performed RT-PCR to amplify the partial ORF of *Gryllus 5-HT_{1A}*, *5-HT_{1B}*, *5-HT_{2α}*, and *5-HT₇*, and obtained a 1386-bp cDNA fragment of *5-HT_{1A}*, a 1038-bp cDNA fragment of *5-HT_{1B}*, a 420-bp cDNA fragment of *5-HT_{2α}*, and a 706-bp cDNA fragment of *5-HT₇* (GenBank accession numbers: AB618098, AB618099, AB618100, and AB618101, respectively). These genes have the following properties: *5-HT_{1A}*, partial ORF = 1–1332 bp, protein product = 443 amino acids; *5-HT_{1B}*, partial ORF = 1–991 bp, protein product = 329 amino acids; *5-HT_{2α}*, partial ORF = 1–420 bp, protein product = 140 amino acids; *5-HT₇*, partial ORF = 17–706 bp, protein product = 230 amino acids (Fig. 5(A), 5(B), 6(A), and 7(A)).

5-HT_{1A} and 5-HT_{1B}: The partial cDNA fragment of both *Gryllus 5-HT_{1A}* and *5-HT_{1B}* receptors encoded the five transmembrane (TM) segments corresponding to the third to seventh TM segments of GPCR (TM3, TM4, TM5, TM6, and TM7 in Fig. 5(A) and 5(B)). The third cytoplasmic loop (the region between TM5 and TM6) of the human *5-HT_{1A}* receptor contains two calmodulin (CaM)-binding sites (Turner *et al.*, 2004). We compared the amino acid

sequence of the third cytoplasmic loop of *Gryllus* 5-HT_{1A} and 5-HT_{1B} receptors and the human 5-HT_{1A} receptor, and found two putative CaM-binding sites in the third cytoplasmic loop of both *Gryllus* 5-HT_{1A} and 5-HT_{1B} receptors (Fig. 5(A) and 5(B)).

Comparison of the deduced amino acid sequence of *Gryllus* 5-HT_{1A} with those of the corresponding part of other 5-HT₁ receptors indicated that *Gryllus* 5-HT_{1A} is closely related to the other known insect 5-HT_{1A} receptors (52.2% identical to *Antheraea* 5-HT_{1A}, 59.3% identical to *Tribolium* 5-HT_{1A}, and 66.8% identical to *Periplaneta* 5-HT_{1A} (Troppmann et al., 2010)). Comparison of the deduced amino acid sequence of *Gryllus* 5-HT_{1B} with those of other 5-HT₁ receptors indicated that *Gryllus* 5-HT_{1B} is structurally most similar to the other known insect 5-HT_{1A} (67.8% identical to *Antheraea* 5-HT_{1B}, 68.0% identical to *Tribolium* 5-HT_{2a}, and 64.1% identical to *Nasonia* 5-HT_{1B}). The molecular phylogenetic analysis of 5-HT₁ receptors using the deduced amino acid sequence of the partial cDNA of *Gryllus* 5-HT₁ receptors and those of the corresponding part of various 5-HT receptors also indicated that *Gryllus* 5-HT_{1A} and 5-HT_{1B} are closely related to the insect 5-HT_{1A} and 5-HT_{1B} receptors, respectively (Fig. 6).

5-HT_{2a}: The cDNA fragment of *Gryllus* 5-HT_{2a} encoded the four TM segments corresponding to the first to fourth TM segments of GPCR (TM1, TM2, TM3, and TM4 in Fig. 7(A)). Comparison of the deduced amino acid sequence of *Gryllus* 5-HT_{2a} with those of other 5-HT₂ receptors indicated that *Gryllus* 5-HT_{2a} is structurally most similar to the other known insect 5-HT_{2a} (80.7% identical to *Drosophila* 5-HT_{2a} (Colas et al., 1995), 87.1% identical to *Tribolium* 5-HT_{2a}, and 87.9% identical to *Apis* 5-HT_{2a} (Thamm et al., 2010)). The molecular phylogenetic analysis of 5-HT₂ receptors using the deduced amino acid sequence of the partial cDNA of *Gryllus* 5-HT_{2a} and those of the corresponding part of various 5-HT receptors also indicated that *Gryllus* 5-HT_{2a} is closely related to the insect 5-HT_{2a} proteins (Fig. 7(B)).

5-HT₇: The cDNA fragment of *Gryllus* 5-HT₇ encoded the five TM segments corresponding to the first to fifth TM segments of GPCR (TM1, TM2, TM3, TM4, and TM5 in Fig. 8(A)). Comparison of the deduced amino acid sequence of *Gryllus* 5-HT₇ with those of other 5-HT₇ receptors, cloned from both vertebrates and invertebrates, indicated that *Gryllus* 5-HT₇ is structurally most similar to the other known insect 5-HT₇ (55.4% identical to *Aedes* 5-HT₇ (Pietrantonio et al., 2001), 70.6% identical to *Tribolium* 5-HT₇, and 66.7% identical to *Apis* 5-HT₇ (Schlenstedt et al., 2006)). The molecular phylogenetic analysis of 5-HT₇ receptors using the partial deduced amino acid sequence of *Gryllus* 5-HT₇ and those of the corresponding part of various 5-HT receptors also indicated that *Gryllus* 5-HT₇ is closely related to the other insect 5-HT₇ receptors (Fig. 8(B)).

Tissue-specific expression patterns of 5-HT-related genes

Tissue-specific expression patterns of 5-HT-related genes were investigated by using RT-PCR analysis. The following ten tissues were subjected to RT-PCR analyses: the central brain, suboesophageal ganglion (SOG), optic lobe (lamina + medulla) + retina, corpus cardiacum-corpora allata complex (CC + CA), thoracic muscles, salivary gland, midgut, Malpighian tubules, testicle, and ovary.

First, we examined tissue-specific expression patterns of genes involved in the 5-HT synthesis (Fig. 9(A)). *Gryllus TRH*, *TPH*, and *AADC* genes showed differential expression levels in different tissues. An intensely stained band of PCR product of *Gryllus TRH* was detected in the lanes of the central brain, SOG, CC + CA, and ovary, whereas that of *Gryllus TPH* was detected in the lanes of the central brain, SOG, CC + CA, thoracic muscle, and salivary gland. A weaker stained band of PCR product of *Gryllus TRH* was detected in the lanes of the

ovary, and that of *Gryllus TPH* was detected in the lanes of the Malpighian tubules, testicle, and ovary. In the lane of the central brain, a 117 bp-longer PCR product corresponding to the *Gryllus TRH* long variant was detected. After extended amplification, PCR product of *TRH* was detected in the lanes of the optic lobe + retina, midgut, and testicle, and that of *TPH* was detected in the lanes of all of the examined tissues (supplementary figure 3). PCR product of *Gryllus AADC* was detected in the lanes of all of the examined tissues.

Gryllus 5-HT receptor genes exhibited receptor subtype-specific distributions (Fig. 9(B)). *Gryllus 5-HT_{IA}* and *5-HT_{IB}*, and *5-HT₇* genes were almost ubiquitously expressed in all of the examined tissues. An intensely stained band of PCR product of *5-HT_{IA}* was detected in the lanes of the central brain, SOG, CC + CA, testicle, and ovary. A weaker stained band of PCR product of *5-HT_{IA}* was detected in the lane of the optic lobe + retina, thoracic muscle, salivary gland, midgut, and Malpighian tubules. An intensely stained band of PCR product of *5-HT_{IB}* was detected in the lanes of the central brain, SOG, CC + CA, testicle, and ovary. A weaker stained band of PCR product of *5-HT_{IB}* was detected in the lane of the optic lobe + retina, thoracic muscle, salivary gland, and midgut. An intensely stained band of PCR product of *5-HT₇* was detected in the lanes of the salivary gland and midgut, and a weaker stained band was detected in the lanes of the other tissues.

The *Gryllus 5-HT₂* gene exhibited a tissue-specific expression pattern. An intensely stained band of PCR product of *5-HT₂* was detected in the lane of the salivary gland, and a weaker stained band was detected in the lanes of the central brain and SOG. In the lanes of the testicle and ovary, slightly stained bands were detected. PCR product of *5-HT₂* was detected in the lane of all examined tissues after extended amplification (supplementary figure 3).

DISCUSSION

5-HT functions as a neurotransmitter/neuromodulator, as a hormone, and as a signaling molecule that regulates segmentation during embryogenesis in insects (Nässel, 1988; Colas et al., 1999). To investigate the molecular basis of the 5-HT system in the cricket, we recently identified three genes involved in 5-HT synthesis (TRH, TPH, and AADC), as well as two 5-HT₁ receptors (5-HT_{1A} and 5-HT_{1B}), one 5-HT₂ receptor (5-HT_{2a}), and one 5-HT₇ receptors in the cricket. We failed to identify the cricket homologue of the β type 5-HT₂ receptor (5-HT_{2 β}). In a further study, we plan to identify and characterize the cricket homologue of the 5-HT_{2 β} receptor.

In insects, genes involved in the 5-HT synthesis were mainly characterized in *Drosophila melanogaster* (Livingstone and Tempel, 1983; Coleman and Neckameyer, 2005; Neckameyer et al., 2007). In *Drosophila*, the first and rate-limiting step of 5-HT biosynthesis is catalyzed by two tryptophan hydroxylases, *dTRH* and *dTPH*, which hydroxylate tryptophan to generate 5-HTP. *dTRH* is primarily expressed in neuronal tissues, and neuronal 5-HT was diminished by null-mutation of *dTRH* (Neckameyer and White, 1992). On the other hand, *dTPH* is thought to be involved in 5-HT biosynthesis in non-neuronal tissues. In the present study, we identified the cricket homologue of *dTRH* and *dTPH*. Interestingly, both *Gryllus TRH* and *TPH* genes were expressed in the CNS, although *Gryllus TRH* is primarily expressed in neuronal tissues while *Gryllus TPH* is more ubiquitously expressed. Our data suggest that TRH- and TPH-mediated 5-HT biosynthesis pathways are not compartmentalized into neuronal and peripheral tissues in the cricket, and the cricket CNS has two distinct mechanisms of the regulation of 5-HT synthesis.

Our expression analysis revealed that mRNA of AADC, which catalyze the second step of 5-HT biosynthesis, was ubiquitously distributed in the cricket. In addition to 5-HT synthesis,

AADC also catalyzes the formation of dopamine, which functions as a neurotransmitter/neuromodulator and is metabolized to produce melanine. Melanin plays essential roles in cuticular sclerotization and the cellular immune response in insects, and *AADC* is up-regulated during the innate immune response in *Drosophila* (Davis et al., 2007). Ubiquitous expression of *AADC* might reflect its functional importance in various biochemical pathways.

With the exception of the 5-HT₃ receptor and the *Caenorhabditis elegans* 5-HT-gated Cl⁻ channels (MOD-1 and LGC-40) (Ranganathan et al., 2000; Ringstad et al., 2009), all of the 5-HT receptors belong to the G-protein-coupled receptor (GPCR) superfamily. GPCR-type 5-HT receptors are one of the most ancient and diversified groups of the ligand-dependent GPCR in animals, and are classified into three subtypes (5-HT₁/5-HT₄/5-HT₅/5-HT₇, 5-HT₂, and 5-HT₆). All known arthropod 5-HT receptors belong to the 5-HT₁/5-HT₇ and 5-HT₂ receptor subtypes. Each 5-HT receptor is coupled with receptor subtype-specific signal transduction mechanisms: Activation of *Drosophila* 5-HT_{1A} and 5-HT_{1B} receptors leads to decrease in cAMP formation and increase in inositol 1,4,5-triphosphate (IP₃) (Saudou et al., 1992). Activation of the *Drosophila* 5-HT₇ receptor leads to increase in cAMP formation. Since the downstream signal transduction of the *Drosophila* 5-HT₂ receptor has not yet been characterized, activation of the receptor might stimulate phospholipase C, resulting in increase in IP₃.

Basically, insects have two 5-HT₁ receptors (5-HT_{1A} and 5-HT_{1B}). Our phylogenetic analysis of 5-HT₁ receptors indicated that the diversification of two insect 5-HT₁ receptor subtypes occurred after the arthropod-nematode divergence (Fig. 6). Interestingly, our data indicated that two *Drosophila* 5-HT₁ receptors structurally resemble each other, and both receptors were classified into the insect 5-HT_{1A} receptor. Two *Drosophila* 5-HT₁ receptor

genes are mapped in adjacent loci (56B1-56B5 on chromosome 2R), and are present in an opposite orientation. Genomic organization of the genes suggests that the *Drosophila* 5-HT_{1A} and 5-HT_{1B} genes were produced by inverted tandem duplication. Moreover, the *Drosophila* genome lacks gene(s) that encode homologue of the insect 5-HT_{1B} receptor subtypes. These data suggest a rapid evolution of the insect 5-HT receptor family in *Drosophila*. Comparative studies of the expression and function of 5-HT receptors may provide insights into the evolutionary modification of the insect 5-HT system.

5-HT is widely distributed in the CNS and in peripheral tissues of both vertebrates and invertebrates. The 5-HT system is involved in modulating various behaviors in the insect CNS, and in the peripheral tissue, 5-HT controls various physiological phenomena such as heart beat, salivary gland secretion, diuresis in the Malpighian tubules (Berridge and Patel, 1968; Collins and Miller, 1977; Maddrell et al., 1991). To elucidate the sites of 5-HT action in the cricket, we investigated the tissue-specific expression patterns of four 5-HT receptor genes in the cricket.

Central nerve system: Our expression analysis revealed that four 5-HT receptors were expressed in the cricket CNS. In the insect CNS, the 5-HT system regulates various behaviors via distinct 5-HT receptor pathways. In *Drosophila*, mutants of the 5-HT_{1A} receptor show a sleep defect, whereas the 5-HT_{1B} receptor is involved in the entrainment of circadian rhythms (Yuan et al., 2005; Yuan et al., 2006). In the *Drosophila* CNS, the 5-HT₂ receptor is expressed in the protocerebrum and ellipsoid body, and is involved in modulating circadian behaviors (Nichols 2007). Moreover, the inhibition of 5-HT₁ receptors and the 5-HT₂ receptor had differential effects on aggressive behaviors (Johnson et al., 2009). In the cricket optic lobe, the 5-HT₇-like receptor is involved in the regulation of circadian pacemaker neurons (Saifullah and

Tomioka, 2003). Functional analysis of each 5-HT receptor in the cricket CNS will reveal the differential involvement of specific 5-HT receptor subtypes to behavior.

Recently, the distribution of 5-HT_{1A} and 5-HT_{1B} receptor proteins were investigated in two cricket species (*Dianemobius nigrofasciatus* and *Allonemobius allardi*) using antibodies produced against the C-terminal Arg/Lys-rich region of the silk moth *Antheraea pernyi* 5-HT₁ receptors (5-HT_{1A} epitope peptide, AFARILFGTHRRGRNKKF; 5-HT_{1B} epitope peptide, RHAFAQRLLCGRRVRRRAPP) (Shao et al., 2010). However, the amino acid sequences of the epitope peptides were quite different from the corresponding parts of *Gryllus* 5-HT_{1A} and 5-HT_{1B} receptors (5-HT_{1A}, AFKRILCGSGRRSRSRKMR; 5-HT_{1B}, RHAFKRILCGRRSARRNRHFGVRYLQ; differential residues are indicated by underlines). Furthermore, the molecular mass of the proteins detected with the anti-*Antheraea* 5-HT_{1A} and anti-*Antheraea* 5-HT_{1B} antibodies were approximately equal to or even smaller than the calculated molecular mass of the partial sequence of *Gryllus* 5-HT₁ receptors which lack the N-terminal extracellular region and first two transmembrane segments (TM1 and TM2). These data raise a question about the reliability of the immunohistochemical assays for the cricket 5-HT₁ receptors in the previous study. Detailed histochemical expression analyses at mRNA and protein levels are needed in further studies.

Corpus cardiacum-corpora allata complex: The corpus cardiacum-corpora allata complex (CC + CA) of crickets contains biogenic amines including 5-HT (Iba et al., 1996). In the cricket *Teleogryllus commodus*, the 5-HT-immunoreactive axons, which presumably originate from pars intercerebralis and pars lateralis of the brain, innervate the CC + CA (Pipa and Moore, 1988). Our data demonstrated that the CC + CA of the cricket express four 5-HT receptor genes, suggesting that the 5-HT system regulates the activity of CC + CA in the cricket. The

amount of biogenic amines in the cricket CC + CA changes in relation to population density (Iba et al., 1995). In the cricket *G. rubens*, a population density-dependent wing polymorphism is regulated by juvenile hormone, and juvenile hormone production in the corpora allata of the larval honeybee is stimulated by octopamine and 5-HT (Rachinsky, 1994). These data allow us to hypothesize that environmental cues (i.e. population density) alter the activity of aminergic systems that affect the activity of the corpus cardiacum-corpora allata complex in the cricket.

Thoracic muscle: Neuromuscular transmission is modulated by 5-HT in both vertebrates and invertebrates. In the lobster neuromuscular junction, 5-HT acts on both presynaptic sites and muscle fibers to facilitate transmitter release, to produce a contracture, and to induce calcium action potentials (Kraits et al., 1980). Expression of 5-HT receptors in the cricket thoracic muscles suggests that the 5-HT-mediated modulation of the skeletal muscle activity is present in the cricket.

Salivary gland: Our expression analysis revealed that the salivary gland of the cricket expresses all four types of 5-HT receptors. In insects, salivation is stimulated by 5-HT, and 5-HT induced secretion is mediated by an increase of intracellular cAMP (Berridge and Patel, 1968; Rietdorf et al., 2005; Troppmann et al., 2007). Of the four 5-HT receptor subtypes, the 5-HT₇ receptor is positively coupled to adenylyl cyclase. Therefore, 5-HT-mediated stimulation of salivation might be mainly mediated by the 5-HT₇ receptor. Further studies are necessary to reveal functional roles of the other three 5-HT receptor subtypes expressed in the salivary gland.

Midgut: A pharmacological study on the locust midgut showed that application of 5-HT leads to relaxation of the midgut circular muscle (Molaei and Lange, 2003). In the midgut of the blood sucking bug *Rhodnius prolixus*, intracellular cAMP was elevated in response to 5-HT (Barrett et al., 1993). Although the pharmacological profile of the 5-HT receptor of the *Rhodnius* midgut

resembled the mammalian 5-HT₂ receptor, the 5-HT₂ receptor is not positively coupled to adenylyl cyclase activation. Our study revealed that the 5-HT₇ receptor, which is positively coupled to adenylyl cyclase, is expressed in the cricket midgut. Therefore, 5-HT-induced elevation of cAMP in the midgut might be mediated by the 5-HT₇ receptor. In mammals, the 5-HT₇ receptor also mediates smooth muscle relaxation in the gastrointestinal tract (Vanhoenacker et al., 2000). 5-HT₇ receptor-mediated regulation of smooth muscle activity in the gastrointestinal tract might thus be evolutionary conserved between vertebrates and invertebrates.

Malpighian tubules: 5-HT acts on the Malpighian tubules as a diuretic hormone in insects (Maddrell et al., 1991). Our expression analysis revealed that the Malpighian tubules mainly expressed 5-HT_{1A} and 5-HT₇ genes. Expression of 5-HT₁ receptors in the Malpighian tubules have also been shown in *Periplaneta* (Troppmann et al., 2010) and *Apis* (Thamm et al., 2010), and expression of 5-HT₇ receptor in the Malpighian tubules have been shown in *Aedes* (Pietrantonio et al., 2001) and *Apis* (Schlenstedt et al., 2006). In the larvae of the mosquito *Aedes aegypti*, 5-HT is released into the hemolymph in response to increased salinity, and stimulates fluid secretion activity of the Malpighian tubules (Clark et al., 1998). In the Malpighian tubules of *Locusta migratoria*, application of 5-HT neither lead to an increase of the intracellular cAMP level nor to an activation of adenylyl cyclase (Morgan and Mordue, 1984). Therefore, 5-HT_{1A}, which is negatively coupled to adenylyl cyclase, might be involved in the 5-HT-mediated stimulation of fluid secretion in the Malpighian tubules.

Testicle and ovary: Almost all of the genes involved in 5-HT biosynthesis and 5-HT receptors were expressed in the testicle and ovary. Serotonergic regulation of the oviduct contraction was reported in several insects (Bamji and Orchard, 1995; Lange, 2004). A pharmacological study on cockroaches revealed that the pharmacological property of the oviduct 5-HT receptor

resembles the vertebrate 5HT₂ receptor (Bamji and Orchard, 1995). Our expression analysis indicated that the cricket ovary expresses not only 5-HT_{2a} but also 5-HT₁ and 5-HT₇ receptors. Our data suggest that 5-HT regulates not only oviduct contraction but also other ovarian activities mediated by the other subtypes of the 5-HT receptors. Moreover, to our knowledge, our study is the first report on expression of 5-HT receptors in the insect testicle. Further studies are necessary to reveal physiological roles of 5-HT on gonadal functions in insects.

In summary, we identified seven genes involved in the synthesis and transduction of 5-HT in the cricket *G. bimaculatus*. Two tryptophan hydroxylase genes, *TRH* and *TPH*, were co-expressed in the cricket CNS, suggesting that the two 5-HT synthesis pathways were not compartmentalized in neuronal and peripheral tissues. Tissue-specific expression analysis of 5-HT receptors showed that the 5-HT system is widely distributed in the cricket, and that the 5-HT system might regulate various aspects of physiological phenomena via distinct 5-HT receptor pathways.

EXPERIMENTAL PROCEDURES

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 *AADC*, *TPH*, *TRH*, and four 5-HT receptor genes

First, we cloned partial cDNAs of *Gryllus AADC*, *TPH*, *TRH*, and four 5-HT receptor genes (*5-HT_{1A}*, *5-HT_{1B}*, *5-HT_{2α}*, and *5-HT₇*) by reverse transcriptase-polymerase chain reaction (RT-PCR) techniques. Next, we performed the 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 and registered their sequences in GenBank.

Complementary DNA preparation for partial cDNA cloning

The cricket brain was isolated in ice-cold phosphate-buffered saline (PBS, pH 7.4). Total RNA was isolated from the brain with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and treated with DNase I (Takara, Shiga, Japan) at 37°C for 1 h. Total RNA was reverse-transcribed using Superscript III reverse transcriptase (Invitrogen) with a random decamer.

Partial cDNA cloning of *TRH*, *TPH*, *AADC*, and four 5-HT receptor genes

TRH: Partial cDNA of *TRH* was amplified using degenerate primers. Degenerate primers were designed on the basis of conserved amino acid sequences (GLAFR and YTPEPD) among the insect TPH proteins. Sequences of the degenerate primers are listed in Table 1.

TPH: Partial cDNA of *TPH* was amplified using GSPs designed on the basis of the *G. bimaculatus* EST clones corresponding to *TRH* (GenBank accession numbers; AK264489, AK268330, and AK270171).

AADC: Partial cDNA of *AADC* was amplified using GSPs designed on the basis of the *G. bimaculatus* EST clones corresponding to *AADC* (GenBank accession numbers; DC443272 and AK278380).

5-HT_{1A}: Partial cDNA of *5-HT_{1A}* was amplified using degenerate primers. Degenerate primers were designed on the basis of conserved amino acid sequences (TLAIITGAFV and WLGYFNST) among the insect *5-HT_{1A}* receptor proteins. Sequences of the degenerate primers are listed in Table 1.

5-HT_{1B}: Partial cDNA of *5-HT_{1B}* was amplified using degenerate primers. Degenerate primers were designed on the basis of conserved amino acid sequences (LAVADLM and DRYWAVT) among insect *5-HT_{1B}* proteins. Sequences of the degenerate primers are listed in Table 1.

5-HT_{2α}: Partial cDNA of *5-HT_{2α}* was amplified using degenerate primers. Degenerate primers were designed on the basis of conserved amino acid sequences (KLQNVTNYFL and LHMCFIGL) among the insect *5-HT₂* receptor proteins. Sequences of the degenerate primers are listed in Table 1.

5-HT₇: Partial cDNA of *5-HT₇* was amplified using degenerate primers. Degenerate primers were designed on the basis of conserved amino acid sequences (LVMPMA and CMISVDR)

among the insect 5-HT₇ receptor proteins. Sequences of the degenerate primers are listed in Table 1.

5' RACE and 3' RACE

To extend partial cDNA clones by amplifying the 5' and 3' sequences of the corresponding mRNAs, we performed 5' and 3' RACEs using the FirstChoice RLM-RACE kit. For the 5' RACE, the cricket brain total RNA was modified using the kit, and was reverse-transcribed using Superscript III reverse transcriptase (Invitrogen) with a random decamer as a primer. For the 3' RACE, the cricket brain total RNA was reverse-transcribed using Superscript III reverse transcriptase (Invitrogen) with a 3' RACE adapter packaged in the kit as a primer. The RACE PCR was carried out using the GSPs designed on the basis of the sequence obtained by the partial cDNA cloning described above.

Complementary DNAs containing the full-length or partial ORF were amplified using the GSPs designed at the 5' and 3' end of the RACE fragments. The primers used to amplify the cDNAs containing the full-length or partial ORF are listed in Table 1.

All PCRs were performed using *LA Taq* polymerase (TaKaRa), *Taq* Blend HighFidelity polymerase (Greiner Bio-One, Tokyo, Japan), *Pfu*-X polymerase (Greiner Bio-One), or Phusion High-Fidelity DNA polymerase (Roche Applied Science, Tokyo, Japan). The amplified DNA fragments were subcloned into the plasmid vector pGEM-T easy (Promega, Madison, WI, USA), and the nucleotide sequences were determined.

Sequence comparison, prediction of transmembrane regions, and phylogenetic analysis

The deduced full amino acids sequences of *Gryllus* TRH short variant, TPH, and AADC, and the deduced partial amino acids sequences of four *Gryllus* 5-HT receptors 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 5.3 program (Drummond et al., 2010). Transmembrane regions of 5-HT receptors were predicted by the TMHMM v. 2.0 program (Sonnhammer et al., 1998; <http://www.cbs.dtu.dk/services/TMHMM/>). Phylogenetic trees were constructed with the aligned sequences by the bootstrap neighbor-joining algorithms on the Geneious 5.3 program. The obtained tree was visualized with the Geneious 5.3 program. The GenBank accession numbers of the proteins used for the comparison are listed in Table 3.

Tissue-specific expression analysis

RT-PCR analysis was performed to assess the tissue-specific expression of the *Gryllus* *TRH*, *TPH*, *AADC*, and four 5-HT receptor 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) testis, and (10) ovary. Seven days after emergence, adult crickets were dissected in ice cold PBS, and total RNA of each tissue was extracted using the TRIzol reagent (Invitrogen). Then, total RNAs were treated with DNase I (TaKaRa) at 37°C for 1 h. 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). For first PCR amplification, 0.2 μ l of cDNA solution was added to a 10 μ l PCR reaction. For extended amplification (secondary PCR), 0.4 μ l of the first PCR product was added to a 10 μ l 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 ethidium bromide. *Ef1alpha*, β -actin, and *rp49* genes were amplified as internal control genes. To confirm that genomic DNA was not present in cDNA solutions, we amplified *Ef1alpha*, β -actin, and *rp49* genes 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.

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FIGURES AND TABLES

Figure 1

5-HT synthesis pathway in insects. 5-HT is synthesized from L-tryptophan in two reaction steps. Tryptophan hydroxylase (*TRH*) and phenylalanine-tryptophan hydroxylase (*TPH*) convert L-tryptophan to 5-hydroxy-L-tryptophan (5-HTP), and aromatic L-amino acid decarboxylase (AADC) converts 5-HTP to 5-HT. The TRH pathway and TPH pathway are compartmentalized in neuronal and peripheral tissues in *Drosophila* (Neckameyer et al., 2007).

Figure 2

Comparison of amino acid sequences of *Gryllus* TRH and TPH with their *Drosophila* homologues. (A) Alignment of the deduced amino acid sequence of *Gryllus* TRH short variant with that of *Drosophila* TRH. (B) Alignment of the deduced amino acid sequence of *Gryllus* TPH with that of *Drosophila* TPH. 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.

Figure 3

Molecular phylogenetic tree of *Gryllus* TRH and TPH with other aromatic amino acid hydroxylases. Insect and vertebrate tyrosine hydroxylases (TH) are used as an outgroup. The

scale bar indicates 0.06 substitutions per site. The GenBank accession numbers of the proteins used for phylogenetic analysis are listed in Table 2.

Figure 4

Comparison of amino acid sequences and phylogenetic tree of *Gryllus* AADC including other AADC proteins. (A) Alignment of the deduced amino acid sequence of *Gryllus* AADC with that of *Drosophila* AADC. 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 putative catalytic loop. The pyridoxal 5'-phosphate binding sites are indicated by black arrowheads. (B) Molecular phylogenetic tree of *Gryllus* AADC and other amino acid decarboxylases. *Drosophila* glutamate decarboxylases (GAD1 and GAD2) are used as an outgroup. The scale bar indicates 0.2 substitutions per site. The scale bar indicates 0.09 substitutions per site. The GenBank accession numbers of the proteins used for phylogenetic analysis are listed in Table 2.

Figure 5

Comparison of amino acid sequences of two *Gryllus* 5-HT₁ receptors (5-HT_{1A} and 5-HT_{1B}) with other 5-HT₁ receptors. (A) Alignment of the deduced amino acid sequence of *Gryllus* 5-HT_{1A} with that of *Antheraea* 5-HT_{1A}. (B) Alignment of the deduced amino acid sequence of *Gryllus* 5-HT_{1B} with that of *Antheraea* 5-HT_{1B}. Identical amino acids are printed in white letters on a black background. Black lines indicate the transmembrane segments (TM). Putative calmodulin (CaM)-binding sites are surrounded by a gray box. The GenBank accession numbers of *Antheraea* 5-HT₁ receptors are listed in Table 2.

Figure 6

Molecular phylogenetic tree of *Gryllus* 5-HT₁ receptors and other 5-HT₁ receptors. *Drosophila* 5-HT₂ and 5-HT₇ receptors are used as an outgroup. The scale bar indicates 0.08 substitutions per site. The GenBank accession numbers of the proteins used for phylogenetic analysis are listed in Table 2.

Figure 7

Comparison of amino acid sequences and phylogenetic tree of *Gryllus* 5-HT_{2α} receptor including other 5-HT₂ receptors. (A) Alignment of the deduced amino acid sequence of *Gryllus* 5-HT_{2α} with that of *Drosophila* 5-HT_{2α}. 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* 5-HT_{2α} receptor and other 5-HT₂ receptors. *Drosophila* 5-HT₁ and 5-HT₇ receptors are used as an outgroup. The scale bar indicates 0.08 substitutions per site. The GenBank accession numbers of the proteins used for phylogenetic analysis are listed in Table 2.

Figure 8

Comparison of amino acid sequences and phylogenetic tree of *Gryllus* 5-HT₇ receptor including other 5-HT₇ receptors. (A) Alignment of the deduced amino acid sequence of *Gryllus* 5-HT₇ with that of *Aedes* 5-HT₇. Identical amino acids are printed in white letters on a black background. Black lines indicate the transmembrane segments (TM). The putative calmodulin (CaM)-binding site is surrounded by a gray box. (B) Molecular phylogenetic tree

of *Gryllus* 5-HT₇ receptor and other 5-HT₇ receptors. *Drosophila* 5-HT₁ and 5-HT₂ receptors are used as an outgroup. The scale bar indicates 0.09 substitutions per site. The GenBank accession numbers of the proteins used for phylogenetic analysis are listed in Table 2.

Figure 9

Tissue-specific expression patterns of *Gryllus TRH*, *TPH*, *AADC*, and four 5-HT receptor genes. (A) RT-PCR analysis for *Gryllus TRH*, *TPH*, and *AADC* genes. (B) RT-PCR analysis for *Gryllus 5-HT_{1A}*, *5-HT_{1B}*, *5-HT_{2a}*, and *5-HT₇* genes. *Gryllus Eflalpha* gene was used as a internal control gene (see supplementary figure 2). SOG = suboesophageal ganglion; CC + CA = corpus cardiacum-corpora allata complex.

Table 1. List of primers used in this study

Degenerate primers		
#	Forward primer	Reverse primer
<i>TRH</i>	5'-GGVYTVGCNTTCCG-3'	5'-CBGGTTCNGGHGTGTA-3'
<i>5-HT_{IA}</i>	5'-GCNATNATYACNGGNGCNTTCG-3'	5'-GTSGAGTTRAARTANCCNARCCA-3'
<i>5-HT_{IB}</i>	5'- GYTNGCCGTNGCNGACYTGATGGT-3'	5'-GTNACNGCCCAGTANCKGTC-3'
<i>5-HT_{2a}</i>	5'- RGCTGCASAAYGTSACYAAYTACTTY CT-3'	5'-AGGCTGATGAARCACATGTGC-3'
<i>5-HT₇</i>	5'-CTNGTNATGCCVATGGC-3'	5'-GGTCSACRSWKATCATGC-3'
Primers for full-length/partial ORF amplification		
#	Forward primer	Reverse primer
<i>TRH</i>	5'-GGGCCAGCAGCGATGAGC-3'	5'-GCTCCCGCAGAGAAGC-3'
<i>TPH</i>	5'-CCCGCATTGTTGGGAGAG-3'	5'-GGTGAACAGAGTAGAGAAATG-3'
<i>AADC</i>	5'-CCGACCCCGTCATGGAG-3'	5'-CCTCTGCAACAGTGATGC-3'
<i>5-HT_{IA}</i>	5'-GGATGGATCCTGGGGCCGGAG-3'	5'- CTGCTACACATATTATGTTAAATT GTTTATTG-3'
<i>5-HT_{IB}</i>	5'-GCTGGGTGCAGTGTACGAG-3'	5'-CCCAGCTGGAACCAGAG-3'
<i>5-HT_{2a}</i>	5'-TACGACTGGAGCTTCCTC-3'	5'-GAGCACGGTATGGACG-3'
<i>5-HT₇</i>	5'-CCACCCCTCCACGCGTATG-3'	5'-GCCGTTGCGCACGCTGATC-3'
Primers for tissue-specific expression analysis		
#	Forward primer	Reverse primer
<i>TRH</i>	5'-CAGCGGGTACTGATGTATGG-3'	5'-GCCCCACGTCTTGATCTC-3'
<i>TPH</i>	5'-GAGGCCGTTGAACCAGC-3'	5'-GGGCGAGGTATTGTATGTGC-3'
<i>AADC</i>	5'-CCCGATTACAGACATTGGCAG-3'	5'-GGCCCATTGTAACCTCTCC-3'
<i>5-HT_{IA}</i>	5'-CTCGCCACCTCCAACAACG-3'	5'-GATGACGGCGTCCTTCTG-3'

<i>5-HT_{1B}</i>	5'-GCCGTCGTACCGTCATCG-3'	5'-CGAGTCCGTTGGCGAAG-3'
<i>5-HT_{2α}</i>	5'-GGCTTCCTGGGGTACTG-3'	5'-GCCGCTTGGTGCTGTAG-3'
<i>5-HT₇</i>	5'-GCTGGAGTACGGCGTGAAG-3'	5'-GGTGAGCGGGATGTAGAAG-3'
<i>β-actin</i>	5'-GGGCCAGAACGGACAGCTATG-3'	5'-CTCCTCAGGGGCAACTC-3'
<i>Ef1alpha</i>	5'-GTGTTCTGAAGGCCAGGTATGG-3'	5'-CTCCAGCAACATAACCACGAC-3'
<i>rp49</i>	5'- GGAAGTGGATGATGCAGAACATCG-3'	5'- CTTCACTGCGTAATCTTGCATTAGC- 3'

Table 2. GenBank accession number of protein sequences used for structural comparison and phylogenetic analyses

		Genes	Species	GenBank accession number
TRH	Insect hydroxylase	tryptophan	<i>Drosophila</i> TRH	<i>Drosophila melanogaster</i> NP_612080
			<i>Apis</i> TRH	<i>Apis mellifera</i> XP_394674
			<i>Tribolium</i> TRH	<i>Tribolium castaneum</i> XP_967413
	Nematoda hydroxylase	tryptophan	<i>Caenorhabditis</i> tph-1	<i>Caenorhabditis elegans</i> NP_495584
	Mollusc hydroxylase	tryptophan	<i>Aplysia</i> TPH	<i>Aplysia californica</i> ABF18968
	Vertebrate hydroxylase 1	tryptophan	<i>Mus</i> TpH1	<i>Mus musculus</i> NP_033440
			<i>Danio</i> TpH1	<i>Danio rerio</i> NP_840091
	Vertebrate hydroxylase 2	tryptophan	<i>Mus</i> TpH2	<i>Mus musculus</i> NP_775567
			<i>Danio</i> TpH2	<i>Danio rerio</i> NP_999960
			<i>Danio</i> tpfd2	AAT38217
TPH	Insect phenylalanin/tryptophan hydroxylase		<i>Drosophila</i> TPH (Henna isoform A)	<i>Drosophila melanogaster</i> NP_523963
			<i>Apis</i> TPH isoform 1	<i>Apis mellifera</i> XP_392163
			<i>Tribolium</i> TPH	<i>Tribolium castaneum</i> XP_967025
	Nematoda phenylalanin hydroxylase	phenylalanin	<i>Caenorhabditis</i> pah-1	<i>Caenorhabditis elegans</i> NP_495863
	Vertebrate phenylalanin hydroxylase	phenylalanin	<i>Mus</i> TPH	<i>Mus musculus</i> NP_032803
			<i>Danio</i> TPH	<i>Danio rerio</i> NP_956845
TH	Insect hydroxylase	tyrosine	<i>Drosophila</i> TH	<i>Drosophila melanogaster</i> NP_476897
			<i>Bombyx</i> TH	<i>Bombyx mori</i> ADV56718
			<i>Apis</i> TH	<i>Apis mellifera</i> NP_001011633
	Vertebrate hydroxylase#	tyrosine	<i>Tribolium</i> TH	<i>Tribolium castaneum</i> NP_001092299
			<i>Caenorhabditis</i> TH	<i>Caenorhabditis elegans</i> ADZ54165
			<i>Mus</i> TH	<i>Mus musculus</i> NP_033403
AADC	Insect aromatic L-amino acid decarboxylase		<i>Danio</i> TH	<i>Danio rerio</i> NP_571224
			<i>Danio</i> TH2	<i>Danio rerio</i> NP_001001829
AADC			<i>Drosophila</i> AADC	<i>Drosophila melanogaster</i> AAO16853

		<i>Aedes</i> AADC	<i>Aedes aegypti</i>	XP_001648263
		<i>Bombyx</i> AADC	<i>Bombyx mori</i>	NP_001037174
		<i>Apis</i> AADC	<i>Apis mellifera</i>	XP_394115
		<i>Tribolium</i> AADC	<i>Tribolium castaneum</i>	NP_001096056
Nematoda	aromatic L-amino acid decarboxylase	<i>Caenorhabditis bas-1</i>	<i>Caenorhabditis elegans</i>	NP_001021150
Vertabrate	aromatic L-amino acid decarboxylase	<i>Mus</i> AADC <i>Danio</i> AADC	<i>Mus musculus</i> <i>Danio rerio</i>	NP_057881 NP_998507
HDC	Histidine decarboxylase	<i>Drosophila</i> HDC	<i>Drosophila melanogaster</i>	NP_523679
TDC	Tyrosine decarboxylase	<i>Drosophila</i> TDC1 <i>Drosophila</i> TDC2	<i>Drosophila melanogaster</i>	NP_610226 NP_724489
Amd	Alpha methyl dopa-resistant	<i>Drosophila</i> Amd	<i>Drosophila melanogaster</i>	NP_724162
GAD	Glutamic decarboxylase	<i>Drosophila</i> GAD1 <i>Drosophila</i> GAD2	<i>Drosophila melanogaster</i>	NP_523914 NP_476788
Insect 5-HT _{1A}		<i>Drosophila</i> HT _{1A} <i>Antheraea</i> 5-HT _{1A} <i>Tribolium</i> 5-HT _{1A} <i>Periplaneta</i> HT _{1A}	5- <i>Drosophila melanogaster</i> <i>Antheraea pernyi</i> <i>Tribolium castaneum</i> 5- <i>Periplaneta americana</i>	NP_476802 ABY85410 XP_967449 CAX65666
Insect 5-HT _{1B}		<i>Drosophila</i> 5-HT _{1B} <i>Antheraea</i> 5-HT _{1B} <i>Tribolium</i> 5-HT _{1B}	<i>Drosophila melanogaster</i> <i>Antheraea pernyi</i> <i>Tribolium castaneum</i>	NP_523789 ABY85411 XP_972856
5-HT ₁	Crustacea 5-HT ₁	<i>Procambarus</i> 5-HT ₁ <i>Penaeus</i> 5-HT ₁	5- <i>Procambarus clarkii</i> <i>Penaeus monodon</i>	ABX10973 AAV48573
Nematoda 5-HT ₁		<i>Caenorhabditis</i> Ser-4 <i>Haemonchus</i> HT ₁	5- <i>Caenorhabditis elegans</i> <i>Haemonchus contortus</i>	NP_497452 AAO45883
Mollusc 5-HT ₁		<i>Aplysia</i> 5-HT _{ap1} <i>Aplysia</i> 5-HT _{ap2} <i>Lymnaea</i> 5-HT ₁ <i>Mizuhopecten</i> HT ₁	5- <i>Aplysia californica</i> <i>Lymnaea stagnalis</i> <i>Mizuhopecten yessoensis</i>	AAC28786 AF372526 Q25414 BAE72141

	Vertabrate 5-HT ₁	<i>Mus</i> 5-HT _{1A} <i>Danio</i> 5-HT _{1A}	<i>Mus musculus</i> <i>Danio rerio</i>	NP_032334 NP_001116793
Insect 5-HT _{2A}		<i>Drosophila</i> HT _{2A}	5-	<i>Drosophila melanogaster</i> NP_730859
		<i>Apis</i> 5-HT _{2A}		<i>Apis mellifera</i> NP_001189389
		<i>Tribolium</i> 5-HT _{2A}		<i>Tribolium castaneum</i> XP_972327
Insect 5-HT _{2B}		<i>Drosophila</i> 5-HT _{2B} (CG42796)	<i>Drosophila melanogaster</i>	NP_649806
		<i>Apis</i> 5-HT _{2B}	<i>Apis mellifera</i>	NP_001191178
		<i>Tribolium</i> 5-HT _{2B}	<i>Tribolium castaneum</i>	EFA04642
5-HT ₂	Crustacea 5-HT ₂	<i>Procambarus</i> HT ₂	5-	<i>Procambarus clarkii</i> ABX10972
	Nematoda 5-HT ₂	<i>Caenorhabditis</i> HT ₂ (Ser-1)	5-	<i>Caenorhabditis elegans</i> NP_001024728
	Mollusc 5-HT ₂	<i>Lymnaea</i> 5-HT ₂		<i>Lymnaea stagnalis</i> AAC16969
Vertabrate 5-HT ₂		<i>Mus</i> 5-HT _{2A}		NP_766400
		<i>Mus</i> 5-HT _{2B}		NP_032337
		<i>Danio</i> 5-HT _{2A}		CAQ15355
		<i>Danio</i> 5-HT _{2A} -like		XP_688270
		<i>Danio</i> 5-HT _{2B}	<i>Danio rerio</i>	NP_001038208
Insect 5-HT ₇		<i>Danio</i> 5-HT _{2C}		NP_001123365
		<i>Drosophila</i> 5-HT ₇	<i>Drosophila melanogaster</i>	NP_524599
		<i>Aedes</i> 5-HT ₇	<i>Aedes aegypti</i>	AF296125
		<i>Apis</i> 5-HT ₇	<i>Apis mellifera</i>	NP_001071289
		<i>Tribolium</i> 5-HT ₇	<i>Tribolium castaneum</i>	XP_966577
5-HT ₇	Nematoda 5-HT ₇	<i>Caenorhabditis</i> 5-HT ₇ (Ser-7)	5-	<i>Caenorhabditis elegans</i> NP_741730
	Mollusc 5-HT ₇	<i>Aplysia</i> 5-HT ₇		<i>Aplysia californica</i> ACJ63458
		<i>Mus</i> 5-HT ₇		<i>Mus musculus</i> CAQ76701
Vertabrate 5-HT ₇		<i>Danio</i> 5-HT ₇ -like 1		XP_690599
		<i>Danio</i> 5-HT ₇ -like 2	<i>Danio rerio</i>	XP_693673

SUPPORTING INFORMATION

Supplementary figure 1

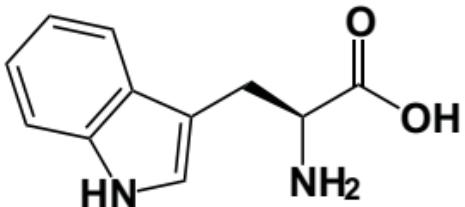
Schematic representation of protein structure of two *Gryllus* TRH isoforms. *Gryllus* TRH long variant contains a 39 amino acid (117 bp) residue insertion within the N-terminal region of the catalytic domain.

Supplementary figure 2

Tissue-specific expression patterns of three house keeping genes (*Gryllus* β -*actin*, *Ef1alpha*, and *rp49*). *Ef1alpha* expression was the one that was most stable in the different tissues.

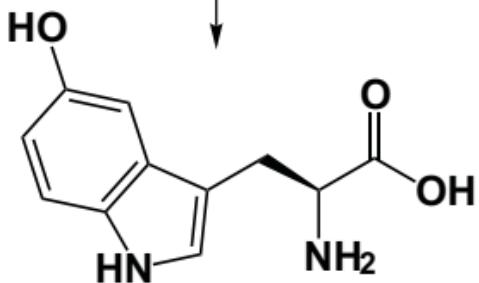
Supplementary figure 3

Tissue-specific expression patterns of *Gryllus* TRH, TPH, 5-HT_{2a} and 5-HT₇ genes. The PCR products of a secondary PCR were run through a 1.5% agarose gel and visualized by ethidium bromide. *Gryllus Ef1alpha* gene was used as a internal control gene.



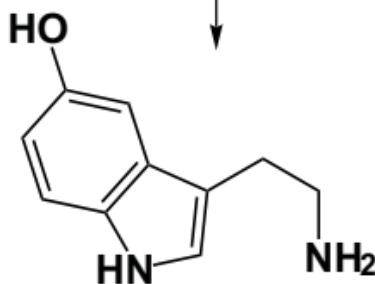
L-Tryptophan

TRH, TPH



5-Hydroxy-L-tryptophan
(5-HTP)

AADC



Serotonin (5-HT)

A

Gryllus TRH 1 MSASGKGLLGLWLYRTGE-GWALKEAPPRAOPPVSVSTCSSGVGVMDHLGK-----DLNSVVFSLRNQVGG
Drosophila TRH 1 MSASGKSLLGLWLYRSGEQEAVKQGSPLHQ---LKKDSTTSGSSSHPS-LGRNASAPPEPPRLAIGGGQDNGRQHSPGERISIIFTLNQVGNL

Regulatory (ACT) domain

Gryllus TRH 68 ARALQVFQDLDVNVLHIESRASSRRDSEFEILVDVQCDERSMSRLVAAIARREVAAINLAHFEGGAALPPPTPLSAASASFDFGEMPWFPKRKISDLD
Drosophila TRH 93 ARALQVFQELCINVLHDELSPLEMATNQADVLDVCDQRRLDQVVKMLNREVASVNNTSNTQGLARAPS-LSACSSDFGDMVWFPRKISDLD

Gryllus TRH 163 DAQRVLMYMGSELDADHPGFKDPVYRKRRREQFSDIANSYKGOPIPRVRQYTPEELKTWTGTVFRELHKLYVKHACOEYLENWPOLVKYCGYREDNIP
Drosophila TRH 187 KAQNVLMYMGSELDADHPGFKDPVYRKRRREQFSAIANNFHGHNPIPRVRQYTPEEVKTWTGTVFELHRLYLVHAVPEYMDNWPELEKYCGYREDNVP

Catalytic domain

Gryllus TRH 258 QLEDVNRFKLCKTGFQLRPVAGYLSPRDFLGLAFRVTFHCTQYIRHSSDPFYTPEPDCCHELLGHMPLLANPSFAQFSQEIGLASLGASDNDIKK
Drosophila TRH 282 QLODVSVYLRKRTGFQLRPVAGYLSPRDFLGLAFRVTFHCTQYIRHSSDPFYTPEPDCCHELLGHMPLLANSFAQFSQEIGLASLGASDADIEK
A A A A A

Gryllus TRH 353 LATLYFFTVEFGLCKO-DGQLRVYGAGLSSVAELRHAVSAAHKTRKFDPDVTCREECIITAFQNAYYTDSFEEAKDKMRDFAGRIOQRPFGVRY
Drosophila TRH 377 LATLYFFTVEFGLCKOADSTFKVYGAGLSSVAELQHAIITAENKIKRFDPETVCQECIITSYQNAYYTDSFEEAKEQMRAFAESIQRPFGVRY
A A A

Gryllus TRH 447 NPYTQSVEVLNSNAEKIAALVSELRGDLICIVSNALRKIHEQDETVDVASLTSMLHKGIELDPSAASAAPHQDGCSAAAASAPSSQPD-SPNPQ---
Drosophila TRH 472 NPYTMSVEVLNSNAKKITAVSELRGDLISIVCSALRKISATDENLDVDSSIANMLHNSLNV-----RGCGASGGGS-BCS-PDNSDNSTAE

Coiled-coil region

Gryllus TRH 554 --
Drosophila TRH 554 GD

B

Gryllus TPH 1 MGIADITEDPLGEYEYID-FILMKGGNYIKEGRD--SAKSTCLIFSEREED-EVGALARSKMFEERHSVNLLHIESRSSSRLEPNRYEFMVEC-AP
Drosophila Hn isoform A 1 -----MYQRQVSF-DRPTRVEDSAYIVEGVDIKEARNTCLLFSPKDSSLSSGALANILAIKKHDINLVHIESRSSLRVPG-YEEFVEADGK

Regulatory (ACT) domain

Gryllus TPH 91 NGNLGAAVENLQKNSSYPKIISRNEKDNRTVWPWFPRRIRDLDKFANQILSYCAELDSDHPGFTDPVYARRKYFADIAYNYKHGEPLPHVDYTE
Drosophila Hn isoform A 86 SGALGKAIEDVKEQCSYFNIIISRDKNDATAVWFPRRIRDLFANQILSYCGELDAHPGFTDPYRKRKYFADIAYNYKHGEPLPHVDYK

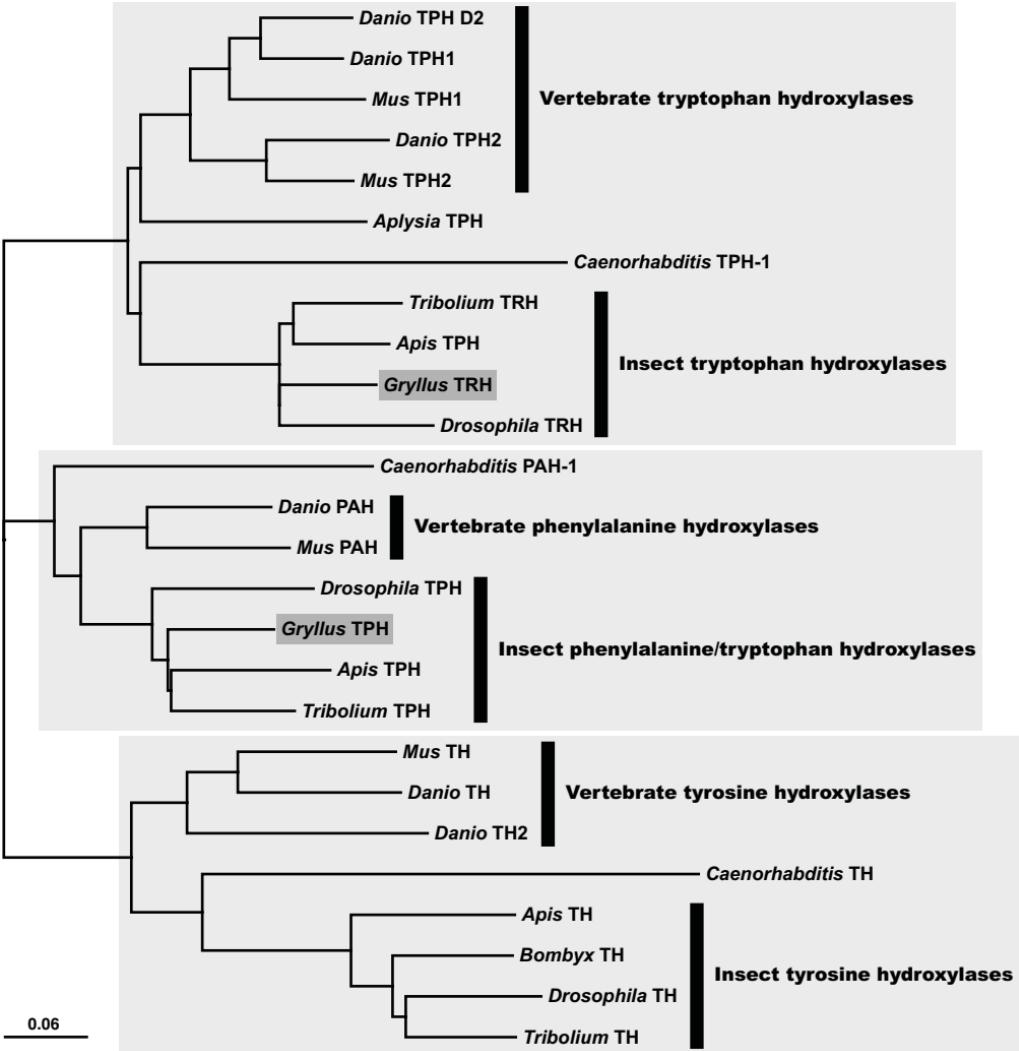
Gryllus TPH 186 EEIETWGHVFRELTKLVPTHACREYNHVFPILLENGFRDENIPQLEDVSNFLRDSTGFSLRPVAGLSSSRDFLAGLAFRVFHCTQYIRHSKSP
Drosophila Hn isoform A 181 EEIETWGIIFRNLTKLVPTHACREYNHVFPILLDNCGFREDNIPQLEDVSNFLDCTGFLRPVAGLSSSRDFLAGLAFRVFHSTQYIRHPSKP
A A A

Catalytic domain

Gryllus TPH 281 YTPPEPDVCHELMGHVPLFADPAFAQFSQEIGMASLGAPDDYIEKLATCFWFTAEGYGLCKODGQLKAYGAGLSSPGELOYCILSDKPELRLPPEPAK
Drosophila Hn isoform A 276 YTPPEPDVCHELMGHVPLFADPAFAQFSQEIGASLGAPDDYIEKLSTIFWFTVEYGVCRQEGELKAYGAGLSSYGELEYCILTDKPOLKDFEPEV
A A A

Gryllus TPH 376 TALQKYPITEYQPVYFVAESFENAKEKMIKWAHTIPRPFGVRYNAYTQSIEIILDSKPQIEPNLVDGHHHEMQILLNALKKL--
Drosophila Hn isoform A 371 TGTVTKYPITQOPLYYYVADSFETAKEKTIKFANSIPRPFGVRYNAYTQSVEVLDSKPQISNLMDNINSEFOIQLONAVAKLRV

Coiled-coil region



A

Gryllus AADC 1 -----METKQFKFKEFARMVDMFGNYLDNIRDPRVLPNVKPGYIREDLPLPESAPEOPEKQDVMADV
Drosophila AADC 1 MSHIPISNTIPKQTDGNGKANISPDKLDPKVSIDMEAPEFKDFAKTMVDFIAEYLENIRRRVLPDVVKPGYIKP~~I~~PDAAPEK~~O~~PEKQDVMADV

Gryllus AADC 61 ERUIMPVGTHHHSPIFHAYFPTANSYPAIVADMGSAGAIACIGFWIATSPACTELEVVMDWLGKMLELP~~EFLAC~~SGKGCCGVIQCTASESTLVAA
Drosophila AADC 96 ERUIMPVGTHHHSPIFHAYFPTANSYPAIVADMGSAGAIACIGFWIATSPACTELEVVMDWLGKMLELP~~EFLAC~~SGKGCCGVIQCTASESTLVAA

Gryllus AADC 156 LLGAKARVPLRKARQENPDVNENDPSKLVLGYIASQAHSSVERAGLLGGVKLRLLPDTANNRRLRADAFQDAIRS~~D~~RQOGLIPPFYAVATLGT~~T~~SSCA
Drosophila AADC 191 LLGAKAKRKEVKELHPWEWDHT~~I~~LGKLVLGYCSDQAHSSVERAGLLGGVKLRSVSE-NHEMRGAALERK~~A~~IEQDVAEGLIPPFYAVATLGT~~T~~NSCA

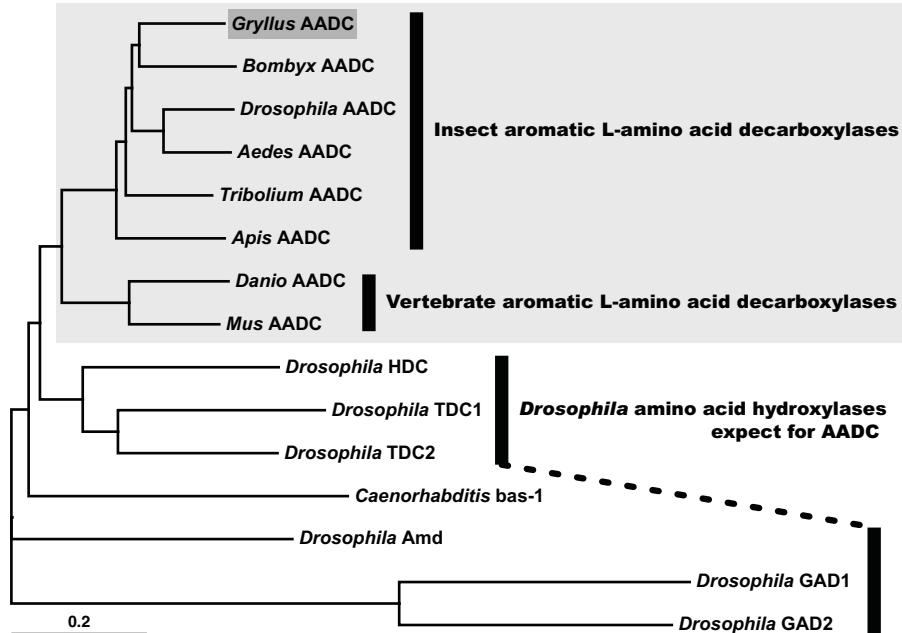
Catalytic domain

Gryllus AADC 251 FDPLEBGLGVVCNQEGW~~H~~WHDVDAAYAGSAFICPEYR~~L~~MAGIE~~S~~ADSFNFNP~~H~~KWLVLNFDCSAMWLKD~~P~~NDDV~~S~~AFNVDPLYLKHD~~O~~QGSAPD~~Y~~
Drosophila AADC 285 FDPLDBCGPVGKHNLW~~H~~WHDVDAAYAGSAFICPEYR~~L~~MAGIE~~S~~ADSFNFNP~~H~~MLVLFDCSAMWLKD~~P~~SWVV~~N~~AFNVDPLYLKHD~~M~~QGSAPD~~Y~~

Catalytic loop

Gryllus AADC 346 HWQIPLGRRFRALKLWFV~~P~~RLY~~G~~IANLOAHIRROIALAHE~~F~~EDHVKS~~D~~SR~~F~~RYCEV~~T~~MGLVCFRLKGSNELNETLLRRINGHGW~~I~~HLP~~S~~SKIRD
Drosophila AADC 380 HWQIPLGRRFRALKLWFV~~P~~RLY~~G~~IANLOAHIRRH~~C~~NFAK~~O~~GDL~~C~~VAD~~R~~FE~~L~~AAE~~I~~MG~~L~~VCFRLKGSNER~~N~~EA~~L~~KKR~~I~~NGRG~~H~~IHLVP~~S~~KRID

Gryllus AADC 441 TYFLRLAICSRFTPSHDEIKLSWN~~E~~VRSLADEVLAEE~~R~~PGN
Drosophila AADC 475 VYFLRLAICSRFTPSHDE~~M~~YEW~~K~~EV~~S~~AA~~D~~MEQEQ---

B

A

Gryllus 5-HT_{1A} 1 GWILGPELCDMWTS~~DVLCTASILHLV~~IAVDRYWAVTNVDYIHTRNSSRIGTMIVVVWAVALIVSLAPQFGWKDPEYLDRINLQQRYLVSQDI
Antheraea 5-HT_{1A} 126 GWILGPELCDMWTS~~DVLCTASILHLV~~IA~~TDRYWAVT~~DV~~DYI~~HIRNERRIFTMIFLVWGAALV~~VSLAPQLGWKDPDYL~~ARITQQQKCLVSQDL

TM3 TM4

Gryllus 5-HT_{1A} 96 AYQVFATCSTFYVPLL~~VILVLYWKIFQ~~TARKRIHRRQQ--RPTVTDH~~ASAAGRS~~GATNNNAPAGGGAGGGAGAGGGGGASATRRLS~~KRRFH~~
Antheraea 5-HT_{1A} 221 AYQIFATCSTFYVPLA~~VILVLYWKIFQ~~TARRR~~RREQPPRPT~~SADGTTPSGRPVQSARD-----REV-KRRLF

TM5 CaM-binding site 1

Gryllus 5-HT_{1A} 188 RIMSPSKK---SSAAEALVSS~~SLVMVEGQ~~STASVDAVGDEETTKASSDNGVGGDAKDEQHAAAGVVTTAFTIS-KSVEQTGAVGVRLLEGGDGVA
Antheraea 5-HT_{1A} 292 ---NLKKCNQRTRAETLAASLLTEGQSTSTVDTL--DEEP-R-----TTAFTINEK-I-

Gryllus 5-HT_{1A} 279 VAVLAT~~SNNVSPEKSS~~TATT~~TNNGSASHQSHMSDM~~SRVEILQKDAVIQKDGSAGTAPEKS-VATI~~HRRDK~~KESLEAKRERKA~~AKTLAIITGAFVV~~
Antheraea 5-HT_{1A} 339 -----SSVSPEKSS~~STVTN~~-----GSKPEKAI~~VPALSHRE~~KESLEAKRERKA~~AKTLAIITGAFVV~~

CaM-binding site 2 TM6

Gryllus 5-HT_{1A} 373 CWLPFFIMAL~~LIP~~CETCYIS~~SDS~~LQSFFLWLGYFN~~STLN~~PVIYTIFSP~~EFRQAFKRILCGSGRSRSRKMR~~
Antheraea 5-HT_{1A} 395 CWLPFFIMAL~~VMP~~ICQSCVIS~~DYL~~ASFFLWLGYFN~~STLN~~PVIYTIFSP~~DFRQAFARILFGTHRRGRNKKF~~

TM7

B

Gryllus 5-HT_{1B} 1 LGAVYEVS~~QWTLGTEL~~CDMWTS~~DVLCTASILHLV~~IA~~ALDRYWAVTNIDYIH~~ORTARRIGLMI~~LVVWLV~~AILV~~SMAPMFGWKDDGWE~~S~~SRILNE~~
Antheraea 5-HT_{1B} 119 LGAVYEVAQR~~WTLGPEL~~CDMWTS~~GDVLCCTASILHLV~~IA~~ALDRYWAVTNIDYIH~~ARTARRVGLMI~~ACVWIVSF~~EVCI~~APLLGWKDPWNRRVSE~~D

TM3 TM4

Gryllus 5-HT_{1B} 96 QRCL~~VQS~~DL~~SYQIFATTSSFY~~PL~~EVLVLYWRIE~~QTARKR~~I~~RRR--VTASAG-----AAGTTGGIAAAVVT~~VIGRPLPTISETTTA~~
Antheraea 5-HT_{1B} 214 LRCVVS~~QDV~~GYQIFAT~~ASSFY~~V~~PVLVIL~~ILYWRIYQTARKR~~I~~RRR~~RGATARG~~GV~~PPPAPAGG~~ALVAAGGSGGIAAAVVA~~VIGRPLPTISETTTA~~

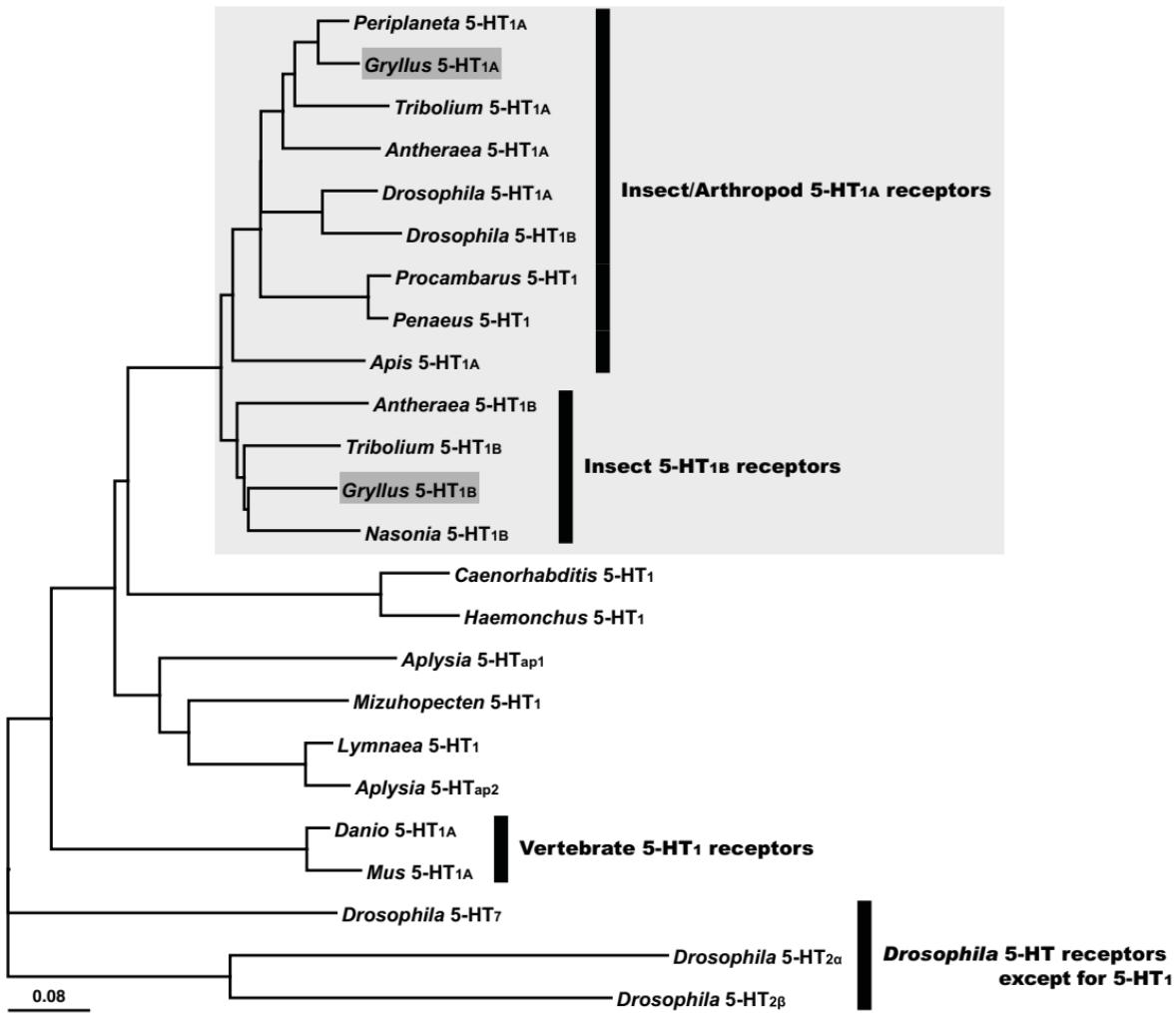
TM5 CaM-binding site 1

Gryllus 5-HT_{1B} 176 -FTTV~~SNTSPEKGSF~~ANGLEP~~DAPT~~VT~~DAGYGAGPA~~AV~~ACPSTPH~~-V~~RRPKD~~STD~~DSK~~RERKA~~AKTLAIITGAFV~~CWLPFFVMAIMMALCGT
Antheraea 5-HT_{1B} 309 GFTNV~~SSNNTSPEKQ~~SS~~ANGLEADP~~PT---TGYG---AVAAAY~~YPTL~~V~~RRPK~~KEA~~ADSK~~RERKA~~AKTLAIITGAFV~~ACWLPFFV~~LAILVPSC~~--

CaM-binding site 2 TM6

Gryllus 5-HT_{1B} 269 SCHLNDL~~VVAVI~~LWLG~~YFNSTLNPIIYT~~FSPEFRHAFKRILCGRR~~SARRRNRF~~GVRYLQ
Antheraea 5-HT_{1B} 395 DCEVSP~~VVLTSLS~~LWLG~~YFNSTLNPIIYT~~FSPEFRHAFKRILCGRR~~VRRRAPP~~-----

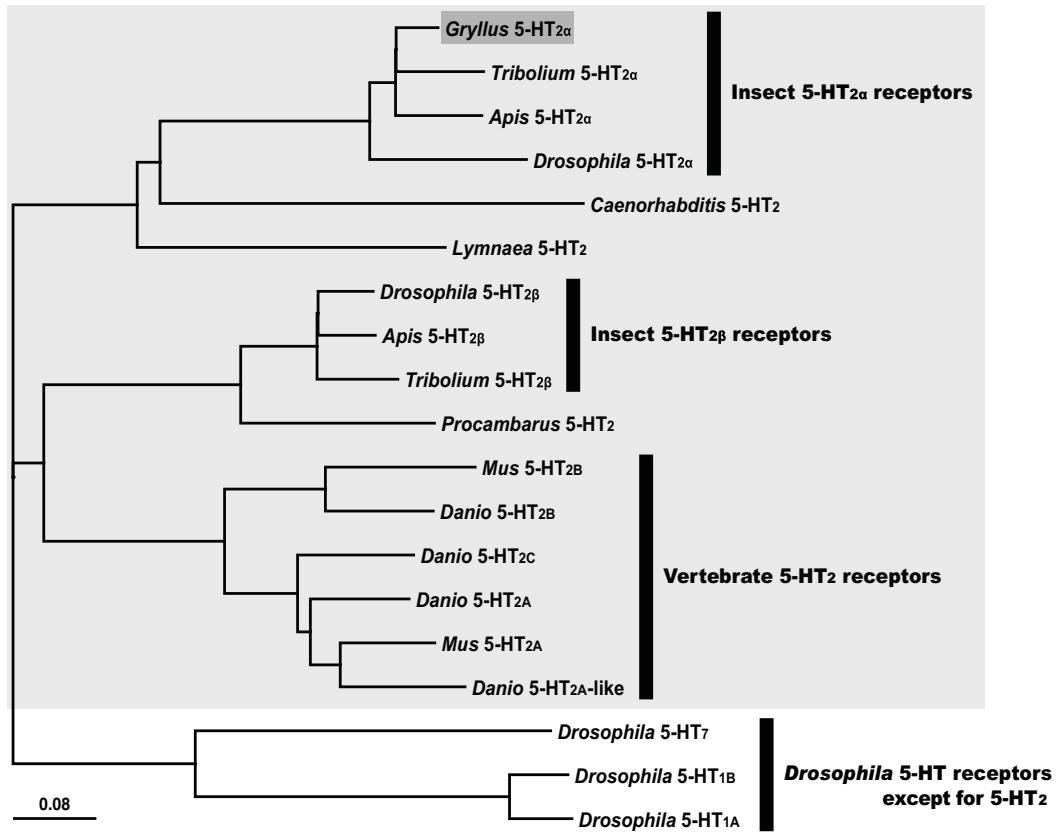
TM7



A

		TM1	TM2	TM3
Gryllus 5-HT _{2a}	1	YDPSLFLFVLF L F I LAGGV G NILVCLAVL G D R KLQNVNTYFL	S L A I A D LLVSLFVMP G A I P G FLG G W P C	V A WCN I YVTCDVLACSSA I MHMC F I
Drosophila 5-HT _{2a}	285	YDPSLFLFVLF V F I AGGV G NILVCLAVL G D R KLQNVNTYFL	S L A I A D LLVSLFVMP G A I P G FLG G W P C	V A WCN I YVTCDVLACSSA I MHMC F I
		TM4		
Gryllus 5-HT _{2a}	96	SLGRY G IRNP L K T RHAY S KTRL V G K IA V W L AM V SSSIT V L		
Drosophila 5-HT _{2a}	380	SLGRY G IRNP L G K R H - S KTRL V G K IA V W L AM V SSSIT V L		

B



A

Gryllus 5-HT₇ 1 -----MEANGTAGLRY-DQG-----
Aedes 5-HT₇ 1 MDPTVFPLLSTLLQQSSAQVLPIDGDPSTVASGVAAIAINATATINIEYLLTGNSSSASVSATAIATSLPALVDRLTPSTSSTSSLDELGGT

Gryllus 5-HT₇ 15 -----ETVAHALVLSCTIVVTIVGNILVCVAVP
Aedes 5-HT₇ 96 SESSPAEPVNVLTIOTIVISIVLLAVIIGTIVGNVLVCVAVC

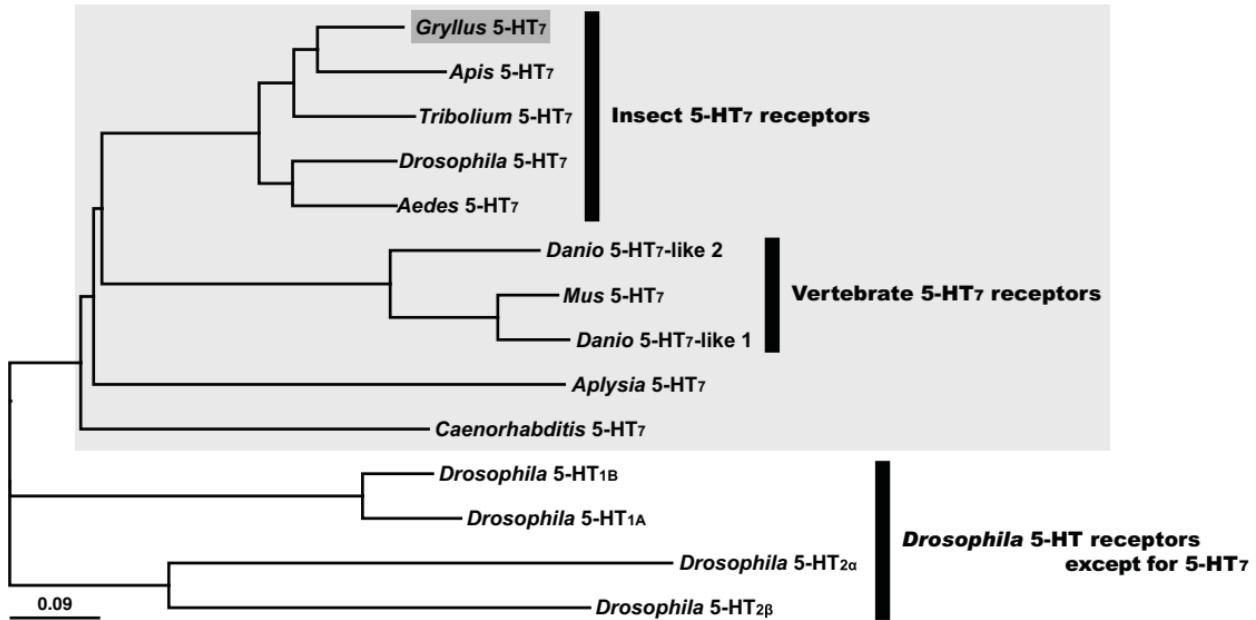
TM1 TM2

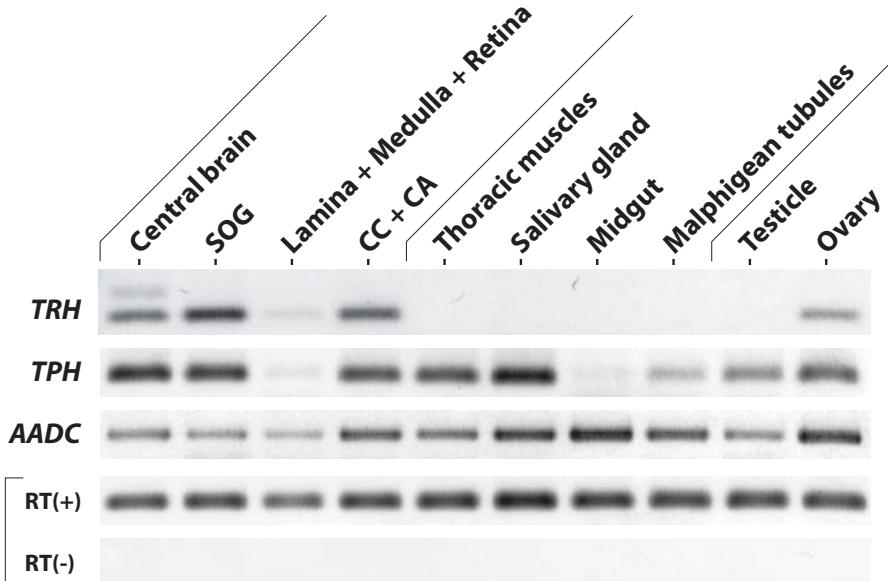
Gryllus 5-HT₇ 96 DVLSCTASILNLCSISVDRYFAITKPLEYGVKRTPRMRVACVALVWLFAACVSLPPLLILGNEHRHEDCSPPCDVCONFQYQIYATLGSFYIPLT
Aedes 5-HT₇ 191 DVLSCTASILNLCSISVDRYFAITKPLEYGVKRTPRMRVACVALVWLVAACVSLPPLLILGNEHRHEDCSPPCDVCONFQYQIYATLGSFYIPLA

TM3 TM4 TM5

Gryllus 5-HT₇ 191 VMEEVVYVKIFRAARRIVMEEERRAQSHLETTHNVEISVRNG
Aedes 5-HT₇ 285 VMLFVYEQIFRAARRIVNEEKRAQKHLETAI-----NG

CaM-binding site

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A**B**