Expression of octopaminergic receptor genes in 4 nonneural tissues in female Nicrophorus vespilloides beetles

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Abstract Octopamine regulates the function of many tissues and physiological processes in invertebrates. The expression of octopamine receptor genes has been examined in multiple tissue types in several different insect orders. However, little work has addressed this issue in Coleoptera. Most studies characterize individual genes in different tissue types, but here we describe the expression of 6 octopamine receptor genes in thoracic musculature, oviducts, Malpighian tubules, and fat body of female Nicrophorus vespilloides beetles to characterize both different genes and different tissues within a single study. We then compare the gene expression profiles found in this beetle to other insects to examine the extent to which expression profiles are conserved across insects. We also examine the relative involvement of octopamine versus octopamine/tyramine receptors based on receptor gene expression in each tissue to help elucidate if tyramine plays a role in the regulation of these tissues. We find a high degree of overlap in the expression profile of the 6 genes examined in the thoracic musculature, a moderate amount for the oviducts, and divergent profiles for Malpighian tubules and fat body. Based on expression differences in receptor subtypes, our results also support the suggestion that tyramine is a biogenic amine with physiological actions separate from octopamine.

Key words biogenic amines; Coleoptera; G-protein coupled receptors; quantitative real-time PCR

Introduction

The octopaminergic system, comprising six receptors and two biogenic amines, plays a fundamental role in the regulation of behavioral and physiological processes in invertebrates (Blenau & Baumann, 2001; Roder, 2005; Verlinden et al., 2010a). Although perhaps best known for its role in behavior, octopamine receptors are expressed in both neural and peripheral tissues and the effect of the octopaminergic system on peripheral tissues may be extensive (Table 1). For example, this system helps regulate energy metabolism, stress responses, immune responses, and ovulation in insects (Roder, 2005; Verlinden et al., 2010a). The octopaminergic system has a well-established direct role in the regulation of muscles (Candy, 1978; Evans & Siegler, 1982), oviducts (Nykamp & Lange, 2000; Lee et al., 2003; Donini & Lange, 2004), Malpighian tubules (Blumenthal, 2003, 2009), and fat body (Wang et al., 1990). Octopamine also acts as a neurotransmitter, neurohormone, and neuromodulator (Roder, 2005; Verlinden et al., 2010a; Farooqui, 2013). The octopaminergic system shares many similarities with the vertebrate adrenergic system by regulating many similar physiological processes, sharing many functional characteristics (Roder, 2005; Verlinden et al., 2010a), and possibly having the same evolutionary origin (Pflüger & Stevenson, 2005; Caveney et al., 2006). Despite the extensive and central role of the octopamine system in behavior and physiology, gene expression in the octopaminergic system is surprisingly poorly characterized outside of a handful of insects (Table 1). In this study we characterized...
gene expression of six octopamine receptors, and quantify expression in multiple tissues. This allows us to compare the profiles of expression in different tissue types, providing insights into possible differentiation of function for the different receptors.

Octopamine is derived from tyrosine and synthesized through a two-step enzymatic process (Blenau & Baumann, 2001; Roder, 2005; Verlinden et al., 2010a). Tyramine was traditionally assumed to only be an enzymatic intermediate in the pathway to synthesize octopamine, but has increasing become regarded as an effector molecule with distinct physiological effects from octopamine (Lange, 2009). There are six established octopaminergic system receptors, which differ in their intracellular signaling properties after activation, homology to vertebrate adrenergic receptors, and affinity for octopamine/tyramine. These receptors have been characterized in different tissues across insects to elucidate how conserved each tissue-specific gene expression profile is across insects.

Here, we investigated the gene expression profiles of receptors in the octopaminergic system in four nonneural tissue types in the beetle *Nicrophorus vespilloides* (Herbst) (Coleoptera: Silphidae), to characterize how genes in this system might influence physiology in a beetle. We have previously characterized these genes, provided a phylogenetic comparison, and examined their expression in neural tissue across different social contexts (Cunningham et al., 2014). The tissue types we investigated here were thoracic musculature (with physiological functions associated with locomotion, flight, thermoregulation), oviducts (protein secretion, egg laying), Malpighian tubules (urine production and ion exchange), and fat body (energy storage and metabolism). We selected these tissues because of their roles in insect physiology and because the octopaminergic system is known to help control their function in at least some insects (Candy, 1978; Evans & Siegler, 1982; Wang et al., 1990; Kutsukake et al., 2000; Nykamp & Lange, 2000; Blumenthal, 2003; Lee et al., 2003; Donini & Lange, 2004; Roder, 2005; Blumenthal, 2009). We had two goals with this study. First, we provide basic information about the octopaminergic system from a beetle in these four tissue types and then compare the gene expression profiles to other insects to elucidate how conserved each tissue-specific gene expression profile is across insects. *N. vespilloides* is a subsocial beetle that has very complex social interactions including extensive parental care. This beetle is therefore potentially very different from the only other well-characterized beetle, the model species, *Tribolium castaneum*. Second, by simultaneously characterizing expression patterns of six receptors in multiple tissues, we predicted we would uncover functional specialization. Other studies often focus on a single tissue or single receptor. As predicted, the most abundant receptor depended on the tissue examined. However, in comparison with other insects, we found that...
expression patterns are conserved for some tissues but variable among taxa for others. Our results also reinforce the view that tyramine has an independent effect from octopamine.

**Materials and methods**

**Insects**

We maintain *N. vespilloides* as an actively outbred colony at the University of Georgia. The founders of this colony were collected from the wild near the University of Exeter, Cornwall in the United Kingdom. See Head et al. (2012) for a description of the population and collecting methods. From dispersal until experimentation, beetles were kept in a common colony room set at 22 ± 1 °C, under a 15 : 9 light : dark cycle, and fed decapitated mealworms (*Tenebrio*) ad libitum once a week after adult eclosion. Beetles were housed individually at dispersal in 5 oz circular deli containers (Eco products, Boulder, CO, USA) filled with 2.5 cm of moist soil.

**Gene expression analysis**

We characterized the expression level of 6 octopaminergic receptor genes: octopamine β-receptor 1 (octβr1; GenBank Accession #: KJ152558), octopamine β-receptor 2 (octβr2; KJ152559), octopamine β-receptor 3 (octβr3; KJ152560), octopamine α-receptor (octar; KJ152561), octopamine/tymarine receptor 1 (tyrr1; KJ152562), and octopamine/tymarine receptor 2 (tyrr2; KJ152563) using quantitative real-time PCR (qRT-PCR) across 4 tissue types. For this we collected samples from thoracic musculature, oviducts, Malpighian tubules, and fat body isolated from virgin females 13 d postadult eclosion. Females are fully capable of successful reproduction at this age. We collected eight biological replicates of Malpighian tubules and fat body and seven biological replicates of thoracic musculature and oviducts.

We submerged whole bodies into ice-cold 1× PBS (National Diagnostics, Atlanta, GA, USA) for tissue collection. After dissection, we submerged tissues into at least 10× their volume of RNAlater (Ambion, Grand Island, NY, USA) on ice and processed the tissues according to the manufacturer’s instructions for storage at −20 °C until RNA extraction.

We used a Qiagen RNeasy Micro kit following the manufacturer’s instructions to extract total RNA with Qiagen QIAzol (700 μL) as the lysis buffer and an addition of 150 μL of chloroform (J.T. Baker, Center Valley, PA, USA) following homogenization. We treated samples with DNase I (Qiagen) to minimize genomic DNA contamination according to manufacturer’s instructions. We quantified RNA using a Qubit 2.0 fluorometer (Invitrogen Corporation, Carlsbad, CA, USA) and synthesized cDNA with Quanta Biosciences qScript reverse transcriptase master mix (Quanta Biosciences, Gaithersburg, MD, USA) using 500 ng of RNA. We stored RNA at −80 °C and cDNA at −20 °C. We made multiple no template controls for each tissue type using the same protocol, except RNase-free water replaced RNA in the cDNA synthesis reaction.

We designed qRT-PCR primers for each gene of interest (GOI) using Primer3 (v4.0.0; Untergrasser et al., 2012) to flank exon boundaries using the draft genome of *N. vespilloides* as a reference and to produce ~100 bp amplicons. Primer information can be found in Appendix 1.

We used Roche LightCycler 480 SYBR I Green Master Mix with a Roche LightCycler 480 (Roche Applied Science, Indianapolis, IN, USA) for qRT-PCR. We ran all samples with 3 technical replicates using 10 μL reactions. Each reaction contained 5 μL of SYBR mix, 2 μL of cDNA diluted 1 : 10 with qRT-PCR grade water, and 3 μL of a 1.33 μmol/L primer stock of both the sense and anti-sense primers. We used the manufacturer’s recommended protocol with an annealing temperature of 60 °C for 45 cycles of amplification. We ran tata-box binding protein (tbp) as an endogenous control gene with each tissue type. We established the stability of *tbp* in a previous study (Cunningham et al., 2014) using the same primer pair and the same experimental methods as in this study. To check for genomic contamination, we ran multiple no template controls of each tissue type with only 2 of 8 samples showing any, and minimal, amplification (1 thoracic musculature sample; 1 Malpighian tubule sample). See Appendix for details.

We used the ΔΔCt method (Livak & Schmittgen, 2001) to examine differences in expression, with *tbp* as our endogenous control gene (Cunningham et al., 2014). We calculated ΔΔCt by taking individual ΔCt values for each gene of interest and subtracting the mean ΔCt value of the most highly expressed gene of interest within a tissue. All ΔΔCt values approached normality. These values were then converted to relative expression by the 2−ΔΔCt method (Livak & Schmittgen, 2001). We visually inspected the data for outliers within technical replicates and removed those that were more than a cycle different from the other values. We tested for the difference in receptor gene expression using an ANOVA. We then used a post hoc Tukey honestly significant difference (HSD) test to assess pairwise significant difference between
Expression of receptor genes of the octopaminergic system, standardized to the mean ΔCt expression of octβ2, in thoracic musculature from Nicrophorus vespilloides adult females. Gene expression was measured using qRT-PCR. Bars are mean ± SEM (n = 7). Letters above bars represent statistically significantly differences using a Tukey HSD post hoc comparison multiple-comparison test. Bars with different letters are statistically significantly different from each other at P < 0.05.

Results

In the thoracic musculature, we found statistically significant differences in the expression of the six different receptor genes (F₅₃₄ = 34.820, P = 0.0001). octβ2 was the most abundant transcript in thoracic musculature followed by tyr1, octar, octβ1, tyr2, and octβ3 (Fig. 1). A post hoc Tukey HSD test showed octβ2 was expressed at significantly greater levels than the other genes (P < 0.0001). tyr1 was expressed at significantly higher levels than tyr2, octβ1, and octβ3.

We also found statistically significant differences in gene expression for the six receptors in oviducts (F₄₃₀ = 14.035, P < 0.0001). octβ2 was again the most abundant transcript in oviducts, with a statistically significantly greater expression than all other receptor genes (Tukey HSD, P < 0.009; Fig. 2). The pattern of the expression of the other genes was different than in the thoracic musculature. In the oviduct, there was no statistically significant difference in the expression of octar, tyr2, tyr1, and octβ1 and octβ3 was not consistently detectable among the biological and technical replicates (Fig. 2).

In Malpighian tubules, there were statistically significant differences in expression (F₃₂₈ = 26.274, P < 0.0001), but this was dominated by the expression of tyr2 and octβ1 and octβ3 were not consistently detectable among the biological or technical replicates (Fig. 3). A post hoc Tukey HSD test showed tyr2 was significantly different from the other genes (P < 0.0001), but no other pairwise comparison was significantly different.

In fat body, we again found statistically significant differences in the expression of the six receptor gene (F₅₃₈ = 3.754, P = 0.0074). octar was the most abundant octopaminergic receptor transcript followed by tyr2 and then octβ2, tyr1, octβ3, and octβ1 (Fig. 4). A post hoc Tukey HSD test showed that only tyr2 was significantly different from octβ1 and octβ3 (P < 0.04).
Fig. 3 Expression of receptor genes of the octopaminergic system, standardized to the mean $\Delta C_T$ expression of tyr2, in Malpighian tubules in Nicrophorus vespilloides adult females. Gene expression was measured using qRT-PCR. Bars are mean ± SEM ($n = 8$). Letters above bars represent statistically significantly differences using a Tukey HSD post hoc comparison multiple-comparison test. Bars with different letters are statistically significantly different from each other at $P < 0.05$.

Discussion

We characterized the expression of six octopaminergic receptor genes in thoracic musculature, oviducts, Malpighian tubules, and fat body of the beetle N. vespilloides. We chose these tissues because they have very different physiological functions and because of the important role the octopaminergic system in regulating their function in other insects (Candy, 1978; Evans & Siegler, 1982; Wang et al., 1990; Kutsukake et al., 2000; Nykamp & Lange, 2000; Blumenthal, 2003; Lee et al., 2003; Donini & Lange, 2004; Blumenthal, 2009). This allows us to make an informal comparison of our results to the expression profiles reported from other insect orders and help address a gap in our understanding about the octopaminergic system across different taxa. Overall, we find similar gene expression profiles for species in octopaminergic receptors in thoracic musculature (flight, locomotion, and thermoregulation) and oviduct (reproduction), while we see more variable gene expression profiles across species in the Malpighian tubules (ion exchange) and fat body (metabolism). We also find that receptor subtypes that have high affinity for tyramine compared to octopamine are more highly expressed in thoracic musculature, Malpighian tubules, and fat body than previously appreciated, along with the moderate role in regulating the oviducts as seen in other species. Our study is also among the first to examine the expression of tyr2 where this pattern is especially evident. These results provide additional support for the emerging consensus of tyramine as a standalone neurohormone.

We found detectable levels of transcripts for all 6 octopaminergic receptors in thoracic musculature of N. vespilloides. Overall, there is a high level of consistency in the expression profiles of octopaminergic genes across several species of insects for this tissue. We found oct$\beta$r2 was the most highly expressed receptor in N. vespilloides, as it is in the lepidopteran Trichoplusia ni (Lam et al., 2013). The orthopteran Schistocerca gregaria has an oct$\beta$r (likely oct$\beta$r2) expressed higher than octar in muscle (Verlinden et al., 2010b), also seen in our results. We found that tyr$\alpha$ was the second highest expressed receptor
gene in muscle in *N. vespilloides*. Functioning *tyrr1* is necessary for proper body wall muscle function in *Drosophila* larvae (Kutsukake et al., 2000), so its expression in muscle is not surprising. A *tyrr* (likely *tyrr1*) is also present in the cockroach *Periplaneta americana* muscle (Rotte et al., 2009); however, very little *tyrr* (likely *tyrr1*) expression was found in *T. ni* muscle (Lam et al., 2013). *octar* was found at moderate levels, consistent with its detectable but not abundant presence in *T. ni* (Lam et al., 2013) and *S. gregaria* (Verlinden et al., 2010b). *octβr1* and *octβr3* were detected at very low levels in this tissue, consistent with expression levels seen in *T. ni* (Lam et al., 2013). We also found low-level expression of *tyrr2*, a receptor type that shows a much greater sensitivity to tyramine compared to octopamine (Cazzamali et al., 2005; Huang et al., 2009), which might suggest a reduced role for tyramine in regulating this tissue compared to octopamine.

Five of the 6 octopaminergic system receptor genes were detectable in the oviducts. *octβr3* was not detectable in *N. vespilloides*, but is detectable in 3 species of Lepidoptera (Lam et al., 2013). As with thoracic musculature, *octβr2* was the most highly expressed receptor gene followed by *octar*. *octβr2* is also the most highly expressed gene in oviducts in *T. ni* (Lam et al., 2013). *octar* is necessary for ovulation in *Drosophila melanogaster* (Lee et al., 2003), so its presence in this tissue is expected. We also note moderate expression of *tyrr2*. Tyramine is suggested to be a cotransmitter with octopamine during ovulation of a locust and *tyrr2* presence in oviduct tissue supports this potential role (Donini & Lange, 2004). *octβr1* and *tyrr1* were found at low levels in oviduct tissue, a pattern also seen with 3 species of lepidopterans (Lam et al., 2013).

Four of the 6 octopaminergic receptors were detectable in *N. vespilloides* Malpighian tubules, with no detectable expression of *octβr1* or *octβr3*. *octβr1* is the most highly expressed octopaminergic receptor gene in the Malpighian tubules of *Trichoplusia ni* and is very highly expressed in two other lepidopteran species (Lam et al., 2013), but had no detectable expression in *N. vespilloides*. *T. ni* has marginally detectable expression of *octβr3* in Malpighian tubules, but levels of *octβr3* are more detectable in the Malpighian tubules in 2 other lepidopteran species (Lam et al., 2013). We also found that *tyrr2* was the most highly expressed gene, in contrast to three species of Lepidoptera where *octβr1* is most highly expressed in Malpighian tubules (Lam et al., 2013). *Bombyx mori* also does not have high levels of expression of *tyrr2* in this tissue (Huang et al., 2009). Tyramine has a well-established role in osmoregulation of *D. melanogaster* Malpighian tubules (Blumenthal, 2003, 2009), which may help explain the high prevalence of *tyrr2* we see in *N. vespilloides*. Octopamine/tyramine receptor gene expression has also been reported in the Malpighian tubules of the rice stem borer *Chilo suppressalis* (likely *tyrr1*; Wu et al., 2013) and the cockroach *P. americana* (Rotte et al., 2009). Low levels of *octβr2* as seen here have also been reported in *C. suppressalis* Malpighian tubules, although the expression of other octopaminergic receptor genes was not measured (Wu et al., 2012). *octar* and *tyrr1* were found at low levels of expression. Overall, our results reinforce the view that tyramine plays a role in the regulation of Malpighian tubule function.

We found that all 6 receptors were detectable in the fat body of *N. vespilloides* with *octar* and *tyrr2* the most highly expressed genes. Our results contrast with the pattern seen in *T. ni* that, with *octβr2* the most highly expressed receptor gene. However, *octar* is also highly expressed in Lepidoptera (Lam et al., 2013). In *S. gregaria*, *octar* is expressed at higher levels than an *octβr2* (Verlinden et al., 2010b), a pattern seen here between *octar* and all of the *octβr*s. *octβr2* was also found at very low expression levels in the fat body of *C. suppressalis* (Wu et al., 2012). *octβr1*, *octβr3*, and *tyrr1* were all at low levels of expression in fat body in *N. vespilloides*. Octopamine influences fat body dynamics through two mechanisms. First, it promotes the release of adipokinetic hormone from the corpora cardiaca (Wang et al., 1990). Second, it directly promotes the release of fatty acids from the fat body (Wang et al., 1990). Blocking *Octar*, but not *Octβr*s, activity inhibits octopamine’s direct influence on the fat body (Wang et al., 1990). This is consistent with our finding of *octar*, but not *octβr*s, in high prevalence in this tissue in *N. vespilloides*. The fact that we see a high level of expression of *tyrr2* suggests a role for this amine in regulating fat body activity.

In summary, our results support the idea that there is a high degree of conservation for the gene expression profiles of the octopaminergic receptors in thoracic musculature. Oviducts show moderate conservation in the pattern of expression of octopamine receptors. In contrast, Malpighian tubules and fat body show more divergence between species in which receptor predominates. This suggests that while specialization in the function of receptors might occur, the exact action of each receptor in each tissue type may be species-dependent. Our results also suggest that different octopamine/tyramine receptors might play a prominent role in the regulation of function in oviducts, Malpighian tubules, and fat body.

**Acknowledgments**

Richard Meagher and Elizabeth McKinney provided expert guidance developing the molecular genetic resources.
for our burying beetles. We thank Paola Barriga, Kyle Benowitz, Ashley Duxbury, Lauren Hebb, Elizabeth McKinney, Patricia Moore, and Eileen Roy-Zokan for discussions and/or comments on the manuscript. The University of Georgia’s Office of the Vice President for Research and the Provost of the University of Georgia provided support for this research. MK Douthit was supported by a University of Georgia Center for Undergraduate Research Opportunities summer fellowship.

Disclosure

The authors declare no conflicting interest.

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Appendix

Additional information on qRT-PCR methods and results

Primer sequences

octβr1:
Sense: GGCATCATCGTGTCCGCTTT
Antisense: ACGGATGGTGACTGTAGCA

octβr2:
Sense: TTCGCCATGACCTTCAA
Antisense: CGAATTCCAGACGTCGCACA

octβr3:
Sense: CAACACCGCCCTAAACACGA
Antisense: CCCTCCAGCTCTTCGACTGT

c octα:
Sense: CGCAGGTCAAACGCTTCAGA
Antisense: GGTGAAGAAGGGTAGCCAGC

tyrr1:
Sense: CGGATCCCATAAACTACGCGC
Antisense: GGCCAATCGTTCCAACCGAT

tyrr2:
Sense: GTGTGGATAAGTTCGGCGCT
Antisense: CGTAGCCCGTGTTCTTGTTGT

tbp:
Sense: CACCCATGACTCAGCAGAT
Antisense: ACGTGCATGCAGAGCTATCTT

Nucleic acid extraction

RNA integrity was assessed with agarose gel electrophoresis, stained with ethidium bromide, and visually inspected. This procedure was done when we were first establishing our extraction protocol to ensure high-quality RNA would be available for the reverse transcriptase reaction.

Primer efficiency

Primer efficiencies were estimated using pooled RNA that was prepared in the same way as the experimental samples from all tissue types and a qRT-PCR run of each primer pair over a 4 point, 4 fold dilution series. The comparative ΔΔC_T of Livak and Schmittgen (2001) assumes that all primers have efficiency close to 2. Primer specificity was established by both a melt curve analysis and running a qRT-PCR reaction out on a 1% agarose gel stained with ethidium bromide.

octβr1: 1.98 (r^2 calibration curve = 0.990)
octβr2: 1.96 (r^2 calibration curve = 0.997)
octβr3: 1.93 (r^2 calibration curve = 0.965)
ocαr: 1.97 (r^2 calibration curve = 0.994)
tyrr1: 1.94 (r^2 calibration curve = 0.976)
tyrr2: 1.96 (r^2 calibration curve = 0.996)
tbp: 2.16 (r^2 calibration curve = 0.989)

No template control (NTC) samples

We tested for possible genomic contamination that remained after our DNase treatment when the original protocol was being worked out. As with this study, very little was found. We therefore tested each of the NTC’s of each tissue type with whatever primer we had the most of at the end of aliquoting the plate, generally this was tbp. The results of the NTC’s showed little evidence of genomic contamination. A total of 6/8 samples had no amplification (both fat body samples, both oviduct samples, 1 thoracic musculature, and 1 Malpighian tubule). A total of 1/8 samples had amplification in 2/3 technical replicates. In this sample, which came from thoracic musculature, the NTC C_T values fell >5 cycles later than the C_T values for the primer tested. The remaining sample had inconsistent amplification in all 3 of the technical replicates. The C_T values for the NTC came from Malpighian tubules and were >10 cycles later than the C_T values for the primer tested.