Research Report

Cloning and distribution of a putative octopamine/tyramine receptor in the central nervous system of the freshwater prawn Macrobrachium rosenbergii

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ABSTRACT

There is ample evidence linking octopamine (OA) and tyramine (TA) to several neurophysiological functions in arthropods. In our laboratory we use the freshwater prawn Macrobrachium rosenbergii to study the neural basis of aggressive behavior. As a first step towards understanding the possible role of these amines and their receptors in the modulation of interactive behaviors, we have cloned a putative octopamine/tyramine receptor. The predicted sequence of the cloned OA/TA Mac receptor consists of 1,579 base pairs (bp), with an open reading frame of 1,350 bp that encodes a 450 amino acid protein. This putative protein displays sequence identities of 70% to an Aedes aegypti mosquito TA receptor, followed by 60% to a Stegomyia aegypti mosquito OA receptor, 59% and 58% to the migratory locust TA-1 and -2 receptors respectively, and 57% with the silkworm OA receptor. We also mapped the OA/TA Mac receptor distribution by in-situ hybridization to the receptor’s mRNA, and by immunohistochemistry to its protein. We observed stained cell bodies for the receptor’s mRNA, mainly in the midline region of the thoracic and in the abdominal ganglia, as well as diffuse staining in the brain ganglia. For the receptor’s protein, we observed extensive punctate staining within the neuropil and on the membrane of specific groups of neurons in all ganglia throughout the CNS, including the brain, the midline region and...
neuropiles of the thoracic ganglia, and ventral part and neuropiles of the abdominal ganglia. The same pattern of stained cells was observed on the thoracic and abdominal ganglia in both in-situ hybridization and immunohistochemistry experiments. Diffuse staining observed with in-situ hybridization also coincides with punctate staining observed in brain, SEG, thoracic, and abdominal ganglia in immunohistochemical preparations. This work provides the first step towards characterizing the neural networks that mediate octopaminergic signaling in prawn.

1. Introduction

Crustacean model systems have been used extensively for studying the neural basis of aggressive behavior and the establishment of dominance hierarchies, focusing primarily on the role played by biogenic amines (see reviews by Huber, 2005; Edwards et al., 2003; Kravitz, 2000). However, species such as lobsters and crayfish establish dominance hierarchies based on size and prior experiences, meaning that in their natural habitat status as dominant or subordinate is usually in flux and not set for the long term (Sosa and Baro, 2002). One means to overcome this experimental problem is to use the freshwater prawn Macrobrachium rosenbergii as a model system. This species is similar to other crustaceans in terms of its anatomy, while it establishes fixed dominance hierarchies based primarily on claw type or adult morphotype. Size and prior experience are secondary considerations, becoming primary considerations only among animals of the same morphotype (Barki et al., 1991a,b, 1992). Adult males of M. rosenbergii develop through three different morphotypes, characterized by their claws and behavior. The blue-clawed animals (BC) are dominant, yellow-clawed animals (YC) are the most submissive of the three morphotypes (Kuris et al., 1987; Ra’an an and Sagi, 1985; Ra’an an and Cohen, 1985).

While it has been shown in the crab that hemolymph concentrations of the biogenic amines octopamine, dopamine and 5-HT are higher following fights (Sneddon et al., 2000; Briffa and Elwood, 2007), the sources and mechanisms of the observed increases are not known. To better understand the actions of these biogenic amines on aggressive behavior, the components of their modulatory systems must be first defined in detail. The 5-HT modulatory system has been fairly well characterized in crustaceans (see reviews by Edwards and Spitzer, 2006; Panksepp et al., 2003; Kravitz, 2000; Beltz, 1999), but much less is known about the octopaminergic system.

Norepinephrine has no physiological relevance in protostomes (including insects and crustaceans) and its role is fulfilled by OA, its invertebrate counterpart (Roeder, 2005). In addition to modifying aggressive behavior in crustaceans, it is involved in regulation of the heart beat (Florey and Rathmayer, 1978), setting responsiveness of neurons in the CNS (Glanzman and Krause, 1983), and regulation of sensory receptor cells (Pasztor and Macnillan, 1990), excitation–contraction coupling (Fischer and Florey, 1987), synaptic transmission at neuromuscular junctions (Dudel, 1965), and escape reflexes (Yeh et al., 1996).

Tyramine (TA) is the synthetic precursor of OA and dopamine (DA), and there is evidence that in invertebrates TA is also involved in a variety of physiological processes indepen-
receptors with 55% identity for both, and 54% identity with both the tobacco budworm OA receptor and the yellow swallowtail TA receptor. The lowest identity was shown between the prawn’s OA/TA$_{mac}$ receptor and the fruit fly TA and OA receptors, as well as with the cattle tick OA receptor with 52% identity with all of them.
The putative OA/TA_{Mac} receptor has the typical G-protein-coupled receptor (GPCR) transmembrane signature motifs. The transmembrane regions participate in both ligand binding and receptor activation. The most highly conserved regions are seen in the transmembrane domains particularly the third. As expected, the third intracellular loop in the receptor is least conserved, with an amino acid identity as low as 28% between sequences. The carboxy terminus is characteristically short, and the amino terminus of the receptor is also poorly conserved.

Phylogenetic analysis was used to compare the putative prawn OA/TA_{Mac} receptor to biogenic amine receptors from several arthropod species (Fig. 2). These receptors include the 5-HT, dopamine, and OA families. It has been proposed that in insects the OA receptor family can be further subdivided into α-adrenergic-like, β-adrenergic-like, and OA/TA type receptors (Evans and Maqueira, 2005). We therefore included representative members from each of these families. Among these closely related receptors, the prawn OA/TA_{Mac} sequence clusters with the octopamine/tyramine family, providing strong support for it being an octopamine/tyramine type receptor.

### 2.2. OA/TA_{Mac} distribution in the CNS of the prawn

OA/TA_{Mac} receptor is widely distributed in the ventral nerve cord. We used in-situ hybridization to localize the receptor’s mRNA in the CNS. It was observed as diffuse staining in the brain and thoracic ganglia, and also as clusters of small- (~10 μm), medium- (~30 μm), and large-sized (~60 μm) cells within the thoracic and abdominal ganglia (n = 18). For these experiments, we designed a DIG-labeled probe for the full length of the receptor and found that there are several groups of cell bodies through the CNS that appear to synthesize the receptor in the prawn (Fig. 3). In the brain, OA/TA_{Mac} receptor’s mRNA was observed as diffuse staining throughout the neuropil areas of the protocerebrum, deutocerebrum and tritocerebrum (Fig. 3A). Between the subesophageal (SEG) and the first thoracic (T1) ganglia, there is a central cluster of small-, medium-, and large-sized cell bodies that showed different intensities of OA/TA_{Mac} staining (Fig. 3B). In the thoracic ganglia, we found from two to four stained cell bodies along the ventral midline, and a group of cells in the ventral midline region of the

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**Fig. 2** – Phylogenetic tree. The evolutionary relationship of the prawn’s putative OA/TA_{Mac} receptor to characterized biogenic amine receptors from other arthropod species indicates it belongs to the octopamine/tyramine family. See Experimental procedures for the details of tree construction and sequence. Numbers at branches represent support values and the branch-length scale bar represents 0.1 amino acid substitutions per site.

**Fig. 1** – Alignment of OA/TA_{Mac} receptor. The amino acid sequences of OA/TA_{Mac} are aligned with homologous invertebrate species. Heavy lines and Roman numbers above the sequence indicate transmembrane regions 1–7. Black boxes with white letters represent areas of identical regions, dark gray boxes with black letters represent conserved regions, and light gray boxes with black letters represent blocks of similar regions. Accession numbers are: cabbage moth Mamestra brassicae, AAK14402; tobacco budworm Heliothis virescens, Q25188; silkworm Bombyx mori, NP_001037504 and BAD11157; fly Drosophila melanogaster, AAA28731 and NP_524419; migratory locust LocustYR Migratoria, Q25321 and Q25322; freshwater prawn Macrobrachium rosenbergii, EU233816. Abbreviations: octopamine receptor (OctR); tyramine receptor (TyrR); tyramine receptor 1 (TyrR1); tyramine receptor 2 (TyrR2); tyramine/octopamine receptor (Tyr/Oct).
T3–T5 ganglia (Fig. 3B). Staining was also observed in all the abdominal ganglia (Fig. 3C–H). Cells are arranged in clusters, following a pattern that repeated itself in each of the six ganglia (A1–A6). The pattern consisted of cells located ventrally at the center of each hemiganglion, along with 4 clusters of cells, arranged as the wings of a butterfly, located towards the dorsal lateral-most edges of each ganglion.

We used a polyclonal antibody that recognizes a portion of the OA/TA<sub>Mac</sub> receptor to determine the distribution of the protein and compare it with that of its mRNA. We also observed OA/TA<sub>Mac</sub> receptor immunoreactivity (OA/TA<sub>Mac</sub>-ir) widely distributed throughout the prawn’s ventral nerve cord (n=3 of each morphotype; Figs. 4 and 5).

In the brain, OA/TA<sub>Mac</sub>-ir was observed in the form of punctate staining in the ventral part of the ganglion, in the protocerebrum, lateralmost aspects of the deutocerebrum, and extending towards the inferior aspects of the tritocerebrum (Fig. 4A). Moving more deeply towards the center of the ganglion, punctate staining continues to be observed in the lateralmost protocerebrum, while medium (~30–50 μm) and large-sized (~60–100 μm) immunoreactive cells are found more medially in this region. More dorsally, punctate staining...
varies and is observed around the periphery of cells. Highly immunoreactive structures within the nucleus, most likely the nucleoli, are observed in all stained cells. Bilateral immunoreactive axons are observed in the optic nerves going towards a region of intense punctate staining in the lateral protocerebrum (Fig. 4B). In the dorsal part of the ganglion, protocerebrum punctate staining the bilateral axons are still visible. In addition, smaller-sized (~10–20 μm) cell bodies showing OA/TA_{Mac}-ir are observed in the deutocerebrum (Fig. 4C).

In the circumesophageal ganglia OA/TA_{Mac}-ir was observed in the form of punctate staining towards the center of the ventral (Fig. 4D), middle (Fig. 4E), and dorsal (Fig. 4F) aspects of the ganglia. OA/TA_{Mac}-ir is observed in two small-sized cells in the ventral part of the ganglia (Fig. 4D), and in one to three medium-sized cells on the surface of the ganglia (Fig. 4E–F).

In the region between the neuropil of the SEG and T1, OA/TA_{Mac}-ir was observed in a central cluster of large-sized cells. We also observed punctuate staining in the neuropil areas of all five thoracic ganglia (Fig. 5A). Single large-sized cells are located in the

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**Fig. 4** - OA/TA_{Mac} immunoreactivity (ir) in the brain and circumesophageal (CEG) ganglia. A1–A3: Brain OA/TA_{Mac}-ir was observed as punctate staining in the ventral part of the ganglion (A1), in the protocerebrum, lateralmost aspects of the deutocerebrum, and extending towards the inferior aspects of the tritocerebrum. Moving more deeply towards the center of the ganglion (A2), punctate staining is observed in the lateralmost protocerebrum, while medium- and large-sized cells are found more medially. Highly immunoreactive structures within the nucleus, possibly nucleoli, are observed in all stained cells. Bilateral axons are observed in the optic nerves going towards a region of intense punctate staining in the lateral protocerebrum (white arrows). More dorsally (A3), protocerebrum punctate staining and the bilateral axons (white arrows) are still visible. Smaller-sized cell bodies showing OA/TA_{Mac}-ir are also observed in the deutocerebrum. (B1–B3) In circumesophageal ganglia, OA/TA_{Mac}-ir was observed as punctate staining towards the center of the ventral (B1), middle (B2), and dorsal (B3) aspects of the ganglia. OA/TA_{Mac}-ir is also observed in two small cells in the ventral ganglia (white arrowheads; B1), and in one to three medium-sized cells on the outer surface of the ganglia (white arrowheads; B2–B3). Scale bar = 100 μm.
midline of the ventral nerve cord, at the first and second thoracic levels. The most prominent staining, however, was observed in the T3–T5 ganglia. Each of these ganglia had OA/TA\textsubscript{Mac} staining in large centrally located clusters of multiple-sized cells.

In the abdominal ganglia, staining was observed in the first five ganglia (A1–A5) in cells arranged in clusters, following the “butterfly wing” pattern mentioned above (Fig. 5B–C), while A6 also had a pair of large clusters of 30–60 \(\mu\)m OA/TA\textsubscript{Mac} stained cells and four immunoreactive cells oriented longitudinally, central and inferior of the neuropil area, extending towards the nerve roots (Fig. 5D). In addition, punctate OA/TA\textsubscript{Mac} staining was observed in the neuropil area of all six ganglia (Fig. 5E–G).

In terms of stained cell bodies, the pattern observed with OA/TA\textsubscript{Mac}-ir is similar to that observed for the receptor’s mRNA. However, as would be expected for a technique that targets sites where transcription and synthesis normally occur, we did not see punctate staining in the neuropil region of the ganglia with \textit{in-situ} hybridization, nor in processes along the connectives. Instead, we observed a more diffuse staining in the same regions that showed punctate antibody staining, suggesting that there may be mRNA fragments ready to be transcribed as receptors to then be inserted into membranes at synaptic terminals.

3. Discussion

To better understand the role of octopamine and its receptor(s) in establishing dominance hierarchies and modulating aggression

![Fig. 5 - OA/TA\textsubscript{Mac} immunoreactivity (ir) in the subesophageal (seg), thoracic (t), and abdominal (a) ganglia. (A) OA/TA\textsubscript{Mac}-ir was found in a central cluster of large cells in the inferior portion of the seg, superior to t1. Punctate staining was also found in the neuropil of all five thoracic ganglia. Single OA/TA\textsubscript{Mac}-ir large cells were found in the midline of the ventral nerve cord, at the t1 and t2 levels (white arrowhead). The t3–t5 ganglia showed the most prominent staining in large centrally located clusters of multiple-sized cells. A pair of bilateral axons extending from t1 to t3 was observed (white arrows), crossing the ventral midline towards the contralateral side at the t2 level, and other of their ramifications going towards the central, lateral, and inferior parts of the ganglia at the t3 level (white asterisks). (B–D) In the abdominal ganglia, staining was observed in cells arranged in clusters, following a “butterfly wing” pattern in the first five ganglia (a1–a5), while a6 also had a pair of clusters of large OA/TA\textsubscript{Mac}-ir cells and four additional cells oriented longitudinally, central and inferior of the neuropil area, extending towards the nerve roots. A representation of the ventral part of a1 (B1), a2 (C1), and a6 (D1) is shown. Punctate OA/TA\textsubscript{Mac} staining was also observed in the neuropil area of all six ganglia. A representation of the dorsal part of a1 (B2), a2 (C2), and a6 (D2) showing the neuropil region is shown. Scale bar = 100 \(\mu\)m.]


in crustaceans, we isolated, cloned, and mapped the distribution of a putative OA/TA<sub>Mac</sub> receptor from the prawn’s CNS.

3.1. Cloning of the OA/TA<sub>Mac</sub> Receptor

Octopamine is widely considered as the invertebrate counterpart of norepinephrine (NE), and all of its known effects are similar to those observed for activation of adrenergic receptors in vertebrates. One of the most well-known functions of adrenergic/octopaminergic signaling is the triggering and modulation of the flight or fight response, which is considered as quick adaptation to energy-demanding situations (Roeder, 2005). Stressful stimuli, such as encounters with predators or rivals, induce this metabolic and behavioral adaptation, leading to enhanced energy supply, increased muscle performance, increased sensory perception, and a corresponding behavioral response (Roeder, 2005).

We cloned a putative OA/TA<sub>Mac</sub> receptor from the CNS of the prawn. Vertebrate and invertebrate biogenic amine receptors have been conserved through evolution in terms of their sequences and signaling pathways (Blenau and Baumann, 2001). We were thus able to take advantage of this sequence conservation to clone this putative receptor from the prawn. Based on its sequence, our cDNA clone encodes a seven transmembrane domain receptor that is most likely a G-protein coupled receptor (GPCR).

Phylogenetic analysis of the OA/TA<sub>Mac</sub> receptor demonstrates a close relationship to other OA/TA-like receptors from arthropods. Specific receptors for OA have been identified through the use of pharmacological and biochemical assays in many invertebrate phyla, including arthropods and mollusks (Axelrod and Saavedra, 1977; Harmer and Horn, 1977; Konishi and Kravitz, 1978; Battelle and Kravitz, 1978; Nathanson, 1979; Battelle et al., 1979; Kravitz et al., 1980; Evans, 1981; Harris-Warrick and Kravitz, 1984). In addition, pharmacological studies in insects have provided evidence for the existence of multiple subtypes of OA receptors (Evans, 1980, 1981, 1987; Evans et al., 1988). Even though the number of these identified receptors is growing they are still likely to represent a small fraction of the existing family members. At this time, not enough characterized sequences from across phyla are available to determine a complete evolutionary lineage of the biogenic amine receptors. Recent data suggests that the family is diverse both within and between phyla. Examples of this include the recently identified trace amine-associated receptor family of tyramine-related receptors identified in vertebrates (Hashiguchi and Nishida, 2007) and the appearance of related receptors in sequenced genomes of distant phyla such as Trichoplax adherens (Srivastava et al., 2008).

Based on the close relationship between our protein sequence with that of characterized insect’s OA and TA receptors, we can preliminarily identify it as an OA/TA type, the first known in a crustacean species.

3.2. OA/TA<sub>Mac</sub> distribution in the CNS of the prawn

To better understand the role played by crustacean OA receptors in aggressive behavior and the establishment of dominance hierarchies, we designed a DIG-labeled probe and raised a polyclonal antibody that recognize this receptor’s mRNA and protein, respectively, and used both to determine its CNS distribution in the prawn.

In terms of specific regions within the prawn’s CNS that showed mRNA expression of the OA/TA<sub>Mac</sub> receptor, both diffuse staining and stained cell bodies were observed in the brain, the lower portion of the SEG ganglion, between it and the first thoracic ganglia (T1), in the midline section and roots of the five thoracic ganglia, in bilateral processes extending from T1 to T3 ganglia, and in the six abdominal ganglia. Likewise, OA/TA<sub>Mac</sub> receptor immunoreactivity (OA/TA<sub>Mac</sub>-ir) is observed as extensive punctate staining within the neuropil, and on the membrane of groups of neurons in all ganglia, and a bilateral process between T2 and T3 ganglia.

Diffuse staining observed with the mRNA probe matches with punctate staining observed when using an anti-OA/TA<sub>Mac</sub> receptor antibody. The diffuse staining pattern observed with in-situ hybridization is indicative of the receptor’s mRNA ready to synthesize the receptor at synaptic sites along neural processes or synaptic terminals. Similarly, punctate antibody staining around the periphery of somata and/or along processes may represent functional aggregations of the receptor in regions where it will perform its signaling role. Localization to the cell body may be indicative that functional receptor here may be responsible for mediating signals that result in rapid changes in the transcriptional activity of the cell. Although we observed punctuate OA/TA<sub>Mac</sub>-ir located on the membrane of groups of neurons, we also observed cytoplasmic staining through all ganglia. This may indicate newly synthesized receptor ready for shipping to the cell membrane or vesicles containing recycled receptor. Similar punctate localization of serotonin and other GPCRs in somata has been previously reported for the nervous systems of other species, such as rat, fruit fly and spider (Gérard et al., 1994; Ramaekers et al., 2001; Panek et al., 2003).

On the basis of previous reports observed in two different lobster species (Schneider et al., 1993; Antonsen and Paul, 2001), and observations from our own laboratory (Reyes-Colón et al., submitted), there is a relative small number of octopaminergic neurons in crustaceans. Of these octopaminergic cells, only a single pair is located in both A4 and A5 in the American lobster (Schneider et al., 1993), and no cells are found in the abdominal ganglia of neither the squat lobster (Antonsen and Paul, 2001), nor the prawn (Reyes-Colón et al., submitted). Similarly, only a small number of neurons contain OA in the entire nervous system of different insects (Roeder, 2005). However, they are enough to supply most neuropils of the brain and most parts of the thoraco-abdominal nervous system with fine meshworks of OA-containing dendritic structures (Roeder, 2005).

Octopaminergic cells of the thoraco-abdominal nervous system of insects supply peripheral effector organs with this transmitter (Roeder, 2005). Likewise, OA-neurons are found within the connective tissue sheath of the second roots of lobster thoracic ganglia (Kravitz et al., 1976). These cells can release OA into the hemolymph at two points, and they are likely to serve a neurosecretory function. One site of release is near the point where the hemolymph passes from the ventral sinus to the base of the gills. The second site is close to the lateral
ends of the second roots, where they enlarge to form pericardial organs. The pericardial organs lie within the pericardial sinus and are believed to contain distal endings for at least some OA cells (Evans et al., 1976). Freshly oxygenated hemolymph courses by this second site immediately before it enters the heart to then be distributed to all tissues.

Peripheral targets of amines have been shown to have no serotonergic innervation, and are supplied exclusively via the circulation by means of serotonin-containing neurosecretory neurons (Kravitz, 2000). Thus the fact that we found expression of the OA/TA receptor in regions where there is no apparent OA innervation lead us to believe that this is most likely also true for OA in the prawn. Immunohistochemical studies in the prawn conducted in our laboratory showed a group of octopaminergic neurons between thoracic ganglia three and four in an area surrounding a small artery and are believed to contain distal endings for at least some OA ends of the second roots, where they enlarge to form pericardial organs.

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4. Experimental procedures

4.1. Experimental animals

Male prawns (Macrobrachium rosenbergii) measuring 8–15 cm in length, from eyestalk to telson, were obtained from the Experimental Aquaculture Station of the University of Puerto Rico and/or from private suppliers in the North coast of Puerto Rico. They were maintained in 30-gallon tanks with continuously filtered and aerated water under a 12 h light/12 h dark cycle. Water temperature was maintained at 26–28°C and the pH adjusted to 7.7 (safe range=6.9–8.5). Animals were fed a high protein (~40%) pelleted purina chow once every other day.

4.2. Dissection

Prawns in the inter-molt (C) stage of the molting cycle (Peebles, 1977) were used, with no missing or recently regenerated limbs and no signs of parasitism. They were anesthetized and euthanized by cooling on ice from 10 to 15 min, until they became fully unresponsive (i.e. movement of swimmerets/walking legs and heartbeat stopped). This method is considered acceptable for crustaceans (Coyle et al., 2004), being faster and producing less distress than exposure to chemical anesthetics, which usually require higher concentrations than those used in fish and other vertebrates. It has also been deemed less disruptive of central nervous system function and structure than exposure to hypertonic saline solutions (Battison et al., 2000). After being weighed and measured, the prawns were transected between the thorax and abdomen. Their claws, walking legs, and carapaces were removed and one segment of the animal was placed in cold (4 °C) prawn saline solution (PSS), while the other segment was dissected. The PSS had the following composition in (mM): NaCl 220; KCl 5.5, CaCl$_2$ 13.5; MgCl$_2$ 2.5; Tris 5; pH=7.4 (Miller et al., 1985). The dissection was carried out on ice. The thoracic and abdominal ventral nerve cord and the brain were isolated quickly with forceps by removing all surrounding organs and muscles and cutting all nerves.

For in-situ hybridization experiments, all tissues were immediately treated in Protease (1 mg/mL PSS) at 34 °C for 5 to 10 min, rinsed four times in PSS, and fixed by immersion in freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer saline (PBS) for 3 h. Preparations were washed in 0.1 M PBS three times for 15 min each, and fully desheathed with fine forceps and scissors.

All procedures involving the use of animals were approved by the University of Puerto Rico Medical Sciences Campus Institutional Animal Care and Use Committee prior to the start of the experiments.

4.3. Cloning of OA/TA$_{Mac}$ receptor

Total RNA was extracted from the central nervous system of the freshwater prawn according to procedures previously described by Chomczynski and Sacchi (1987). Briefly, a magnasphere kit (Promega, Madison, WI, USA) was used to isolate mRNA from total RNA. First-strand cDNA was produced in a standard 20-μl reaction using Superscript II (Life Technologies, Gaithersburg, MD, USA) and random hexamer primers. The 1250 bp fragment containing the coding sequence for the OA/TA$_{Mac}$ receptor was amplified by RT-PCR using the primers designed in the present study.
The scientific name, common name, abbreviation, and accession number for all species and sequences used in the phylogenetic analysis are listed as follows: Apis mellifera, Honey bee (DA1, NP_001011595.1; DA2, NP_001011567.1); Bombyx mori, Silkworm (DA1, NP_0010118459.1; DA2, NP_0010118338.1; Tyr, BADD11157; Oct, NP_001037504; 5-HT1, NP_001037502.1; Drosophila melanogaster, Fruit fly (DA1, AAC47161.1; DA2E, NP_001020801.1; Oct-α, AAFF5798.2; Oct-β, AAC66171.2; Tyr, P22270.2; 5-HT1, NP_476802; 5-HT2, NP_649806; DA1, AAC47161.1; DA2E, NP_001020801.1); Heliotis virescens, Tobacco budworm (Oct, Q52188); Locust migratoria, Migratory Locust (TA, Q52321); Macrobriachium rosenbergii, Prawn (5-HT1, EU363466; 5-HT2, EFO33662; OA/TA_mac, EU233816); Mamestra brassicae, Cabbage Moth (Oct, AAK14402); Panulirus interruptus, Lobster (DA1, ABB87182.1; DA1b, ABB87183.1; DA2, ABI64137.1; 5-HT1, AAS18607.1; 5-HT2, AASS7919); Periplaneta americana, Cockroach (Oct-α, AAP98317.1; TA, CAQ48240.1); Procambarus clarkii, Crayfish (5-HT1, ABX10973.1; 5-HT2, ABX10972.1); Schistocerca gregaria, Desert Locust (Oct-α, ADD91574.1; Oct-β, ADD91575.1).

4.4. RACE to obtain the OA/TA_mac receptor termini

We performed 5’ and 3’ rapid amplification of cDNA ends (RACE) using the following RACE primers:

5’ RACE 5’-CCCATCTTGCTCTCCATCCATGCGC-3’
3’ RACE 5’-CCATTCTGCTCCATTCTACGTACGCCG-3’

The prawn’s mRNA was purified from nervous system total RNA with Oligotex (Qiagen, Chatsworth, CA, USA). 5’ and 3’ RACE reactions were performed with the SMART RACE amplification kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer’s instructions. RACE products were cloned with a TA cloning kit (Invitrogen, La Jolla, CA, USA), and sequenced (Cornell BioResource Center, Ithaca, NY, USA).

BLAST analysis followed by paired alignments with invertebrate TYR- and OA-like receptors was conducted to confirm the identity of the prawn’s OA/TA_mac receptor. The prawn’s OA/TA_mac predicted amino acid sequence was then aligned with TYR-, and OA-like receptors from other species using ClustalX (Jeanmougin et al., 1998; Thompson et al., 1997) with default parameters. Accession numbers for sequences used in this alignment were: Macrobriachium rosenbergii, MrTyrOctMac_R, EU233816; Bombyx mori, BmTyrR, BADD11157; BmOctR, NP_001037504; Heliotis virescens, HvOctR, Q25188; Mamestra brassicae, MbOctR, AAK14402; Locust TYZYR migratoria, LmTyrR2, Q25322; LmTyrR1, Q25321; Drosophila melanogaster, DmTyrR NP_524419; DmOctR AAA28731.

4.5. Phylogenetic analysis

The phylogenetic tree was generated using the maximum likelihood algorithm implemented in the program TREE-PUZZLE (Schmidt et al., 2002) model of amino acid substitution, exact parameter estimates, and 10,000 puzzling steps were used in the calculation. The initial multiple alignment was done using ClustalX (Jeanmougin et al., 1998; Thompson et al., 1997) with default parameters. Gaps in the alignment were removed manually in GeneDoc (Nicholas et al., 1997) prior to tree construction. The tree image was first generated using Treeview (Page, 1996) and the final graphic was produced using Corel Draw.
Glycerol Buffer and mounted it in a slide with a cover slip to take pictures with a Zeiss Axioscop microscope. Controls included use of the sense probe instead of the anti-sense, and omission of the probe.

4.7. Generation of affinity-purified anti-OA/TA<sub>Mac</sub> antibodies

The amino acids sequences of OA/TA receptors from the cabbage moth (Accession Number: AAK14402), tobacco budworm (Accession Number: Q25188), silkworm (Accession Numbers: NP_001037504, BAD11157), fly (Accession Numbers: AAA28731, NP_524419), migratory locust (Accession Numbers: Q25321, Q25322), and freshwater prawn (Accession Number: EU233816) were aligned (Fig. 1) and two conserved, highly charged, non-transmembrane regions were identified:

Peptide A CQFIEEKQISLSKERRAART Peptide B DWPDVFETDTPCILTEEKG

To determine if any known proteins other than OA/TA receptors contain a similar peptide sequence, each peptide was used in a protein-protein blast [http://www.ncbi.nlm.nih.gov/blast/Blast.cgi], and no such instances were found. Peptides representing these two regions were synthesized and each was used as an antigen in the production of affinity-purified sheep polyclonal antibodies. For peptide A, a cysteine was included at the beginning of the sequence in order to conjugate it to the carrier, KLH. Peptide synthesis, conjugation to KLH, antibody production, and affinity purification were performed by Bethyl Laboratories (Montgomery, TX, USA).

4.8. OA/TA<sub>Mac</sub> immunoreactivity

Ventral nerve cords were removed in PSS as described above. They were immediately fixed by immersion in freshly prepared 4% paraformaldehyde in 0.1 M PBS at room temperature for 3 h with constant shaking, and rinsed overnight in 0.1 M PBS containing 0.2% Triton X-100 (PBST). Preparations were incubated in normal donkey serum (1:20) in 0.1 M PBST at 4 °C for 3 h, followed by the primary polyclonal sheep anti-OA/TA<sub>Mac</sub>-8 antibody (Bethyl Laboratories, TX, USA), at a concentration of 5 μg/ml in PBST for 2–3 days. After washing 12 times with 0.1 M PBST, 30 min each, tissue was incubated in the secondary antibody donkey anti-sheep Alexa 488 (Molecular Probes, Carlsbad, CA, USA) at a dilution of 1:200 in PBST at 4 °C overnight. Preparations were washed in 0.1 M PBST 6 times, 1 h each, then in 0.1 M PBS 5 times, 15 min each, and left overnight in 90% glycerol/PBS buffer. The next day they were mounted with Polyacryomount, coverslipped, and viewed with a Zeiss Axioskop using epifluorescent excitation and/or a Zeiss LSM Pascal confocal microscope. The brightness and contrast of the digital images obtained by the Zeiss LSM Pascal software were adjusted so that they would be uniform in the figures presented here. Confocal stacks were reconstructed and analyzed in Adobe Photoshop 7.0 on a Dell PC computer. Controls included preadsorption of the primary antibody with the peptide used to generate the antibody for 3–4 h at room temperature before incubation with the preparation (peptide to antibody: 1:20, w/w).


