The Journal of Neuroscience, May 15, 1998, 18(10):3650–3658

A Novel Octopamine Receptor with Preferential Expression in *Drosophila* Mushroom Bodies

Kyung-An Han,[1] Neil S. Millar,[3] and Ronald L. Davis[1,2]

Departments of [1]Cell Biology and [2]Neurology, Baylor College of Medicine, Houston, Texas 77030, and [3]Wellcome Laboratory for Molecular Pharmacology, Department of Pharmacology, University College London, London WC1E 6BT, United Kingdom

Octopamine is a neuromodulator that mediates diverse physiological processes in invertebrates. In some insects, such as honeybees and fruit flies, octopamine has been shown to be a major stimulator of adenylyl cyclase and to function in associative learning. To identify an octopamine receptor mediating this function in *Drosophila*, putative biogenic amine receptors were cloned by a novel procedure using PCR and single-strand conformation polymorphism. One new receptor, octopamine receptor in mushroom bodies (OAMB), was identified as an octopamine receptor because human and *Drosophila* cell lines expressing OAMB showed increased cAMP and intracellular Ca^{2+} levels after octopamine application. Immunohistochemical analysis using an antibody made to the receptor revealed highly enriched expression in the mushroom body neuropil and the ellipsoid body of central complex, brain areas known to be crucial for olfactory learning and motor control, respectively. The preferential expression of OAMB in mushroom bodies and its capacity to produce cAMP accumulation suggest an important role in synaptic modulation underlying behavioral plasticity.

Key words: adenylyl cyclase; single-strand conformation polymorphism; G-protein-coupled receptor; associative learning; octopamine receptor; mushroom bodies; neuromodulation

Neuromodulatory systems play crucial roles for animal behavior by modifying the synaptic output of relevant neurons. Octopamine is a major neuromodulator with neurotransmitter and neurohormone functions that mediates diverse physiological processes in the peripheral nervous system and CNS of invertebrates. It acts as a neurotransmitter for light production in the firefly lantern, has an excitatory modulatory function in the somatic and visceral muscles of locust, and regulates carbohydrate and fatty acid metabolism in locust (David and Coulon, 1985). Female fruit flies that are devoid of octopamine because of a biosynthetic defect display an egg retention phenotype, suggesting a modulatory role of octopamine in oviductal muscular contraction (Monastirioti et al., 1996). Octopamine is also involved in the display of submissive postures in lobsters (Livingstone et al., 1980), escape behavior of crayfish (Glanzman and Krasne, 1983), and feeding behaviors of blowflies (Long et al., 1986) and honeybees (Braun and Bicker, 1992). In *Drosophila*, inactive mutants containing 15% wild-type levels of octopamine display hypoactivity (O'Dell, 1993). Conversely, octopamine application to decapitated flies induces strong stimulation of locomotion and grooming behavior (Yellman et al., 1997). Moreover, crucial roles of octopamine are implicated in complex behaviors, including conditioned courtship and olfactory learning in *Drosophila* (Dudai et al., 1987; O'Dell, 1994). Together, these observations illustrate the diverse functions of octopamine in motor control and behavior. These effects are presumably produced by octopaminergic neurons that are dispersed in the CNS (Monastirioti et al., 1995) acting on receptors at their targets. Although biochemical studies have revealed high-affinity octopamine binding sites (Dudai and Zvi, 1984) and a strong potency of octopamine to stimulate adenylyl cyclase (AC; Uzzan and Dudai, 1982), an octopamine receptor with this activity has not been identified.

The cumulative studies of *Drosophila* learning mutants have revealed two overriding parameters for olfactory learning. First, the cAMP signaling pathway is critical for normal olfactory conditioning. Animals defective in cAMP phosphodiesterase (*dnc*), AC (*rut*), and protein kinase A (*DCO*) are impaired in olfactory learning (Davis, 1996). Second, the mushroom bodies are a major neuroanatomical site for associative learning. Neuroanatomical and physiological studies have shown that mushroom bodies receive diverse sensory inputs (Schürmann, 1987), and flies with malformed or missing mushroom bodies have defective olfactory learning (Heisenberg et al., 1985; de Belle and Heisenberg, 1994). Furthermore, genes critical for learning and memory, *dnc*, *rut*, *DCO*, *leo*, and *Volado*, are predominantly expressed in the mushroom body neuropil (Nighorn et al., 1991; Han et al., 1992; Skoulakis et al., 1993; Skoulakis and Davis, 1996; Grotewiel et al., 1997). In addition, the α subunit of G_s-proteins ($G_s\alpha$) is expressed at a higher level in the mushroom body neuropil (Forte et al., 1993), and the targeted expression of the constitutively active $G_s\alpha$-protein abolishes olfactory learning (Connolly et al., 1996). These observations indicate that mushroom bodies are principal neuroanatomical substrates for olfactory learning and memory and that they use a cAMP-mediated signaling pathway mediated by G-protein-coupled receptors (GPRs) for their physiological modulation. However, the identity of the modulatory neurotransmitter that triggers this biochemical cascade in mushroom bodies remains unidentified.

We report here the isolation of a novel octopamine receptor, octopamine receptor in mushroom bodies (OAMB). This receptor is highly enriched in the mushroom bodies and stimulates

Received Dec. 11, 1997; revised Feb. 25, 1998; accepted March 3, 1998.

This work was supported by grants from the Human Frontiers Science Project, National Institutes of Health, and the R. P. Doherty-Welch Chair in Science to R.L.D. Additional support was from the Wellcome Trust to N.S.M. We thank S. Ahmed and J. Volmer for expert technical assistance. Correspondence should be addressed to Dr. Ronald L. Davis, Department of Cell Biology and Neurology, Baylor College of Medicine, Houston, TX 77030.

cAMP and intracellular Ca^{2+} ($[Ca^{2+}]_i$) accumulation on octopamine application. This is the first octopamine receptor cloned from insects with potency to activate cAMP signaling cascades. The key roles for octopamine in behavioral plasticity, the prominent expression of OAMB in the mushroom body neuropil, and the ability of OAMB to activate AC make this receptor an attractive candidate for mediating the signal transduction cascades underlying learning and memory processes in *Drosophila*.

MATERIALS AND METHODS

Nucleic acids. Primers used for PCR were 5′-TTCGTCATCTGCTGGCTGCCCTTCTTC-3′ and 5′-TGGCTGGGCTACATCAACTCG-3′, corresponding to sequences in transmembrane domains (TMs) VI and VII of the *Drosophila* tyramine receptor (TYR) (Arakawa et al., 1990; Saudou et al., 1990). The cDNA clones corresponding to TYR (Saudou et al., 1990), DRO1 (Witz et al., 1990), DRO2A, and DRO2B (Saudou et al., 1992) were kindly provided by Dr. R. Hen (Columbia University, School of Medicine, New York, NY). cDNAs were synthesized using avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) and random hexamers (Han and Kulesz-Martin, 1992). PCRs were performed with 100 ng of genomic DNA or cDNA in the presence of 5 μCi of [^{32}P]dCTP (Han and Kulesz-Martin, 1992). For single-strand conformation polymorphism (SSCP) analysis, the PCR products were diluted 10-fold in 0.1% SDS and 10 mM EDTA, denatured at 95° for 5 min, and resolved on 4% nondenaturing acrylamide gels (Orita et al., 1989). Isolation of nucleic acids, blotting, cloning, screening, and sequencing were performed using standard procedures.

Pharmacology. A 2461 bp fragment [nucleotides (nt) 919–3380] of OAMB cDNA containing the open reading frame was subcloned into the *Drosophila* expression vector pRmHa3 and transfected into the *Drosophila* S2 cell line (Millar et al., 1994). The same OAMB cDNA fragment was also subcloned into the mammalian expression vector pcDNA1/Amp (Invitrogen, Torrance, CA) and transfected into human HEK-293 cells. Stably transfected S2-OAMB cells and transiently transfected HEK-OAMB cells were assayed for agonist-induced changes in cAMP levels using a [^{3}H]cAMP assay system (Amersham, Arlington Heights, IL) as described by Han et al. (1996). Cells were incubated with agonists in the presence of 0.1 mM 3-isobutyl-1-methylxanthine (an inhibitor of phoshodiesterase) for 15 min at room temperature. In both transfected and untransfected cells, the level of cAMP detected in assay samples was 0.9 ± 0.3 pmol/10^6 cells in the absence of agonist. As in previous studies (Han et al., 1996), we have presented the cAMP assay data as a percentage of the maximal response. The changes in $[Ca^{2+}]_i$ were measured in the HEK-OAMB cells loaded with 4 μM fura-2 AM (Molecular Probes, Eugene, OR) after ligand treatment using a Perkin-Elmer (Emeryville, CA) LS50B fluorescence spectrometer (Cooper and Millar, 1997).

In situ *hybridization and immunohistochemistry.* A clone containing the 5′ half of the OAMB cDNA (nt 1–1614) served to make RNA probes using digoxigenin-UTP (Boehringer Mannheim, Indianapolis, IN). Ten-micrometer frontal sections of Canton-S flies fixed in 4% paraformaldehyde were hybridized with riboprobes and processed for the immunological detection of hybridized transcripts as described by Han et al. (1996).

A 542 bp fragment (nt 1916–2458) of the OAMB cDNA was subcloned in pGEX-KT (Pharmacia, Piscataway, NJ) to generate a fusion protein with the glutathione *S*-transferase. After the third injection of the fusion protein into specific pathogen free rabbit, the antiserum was collected and affinity-purified for immunostaining (Han et al., 1996). Canton-S flies were fixed in 2% paraformaldehyde for 3 hr and soaked in 25% sucrose solution overnight at 4°C. Ten-micrometer cryosections were incubated with the affinity-purified anti-OAMB antibody or the preimmune serum and processed as described by Han et al. (1996).

RESULTS

Isolation of novel biogenic amine receptors

To identify new biogenic amine receptors expressed in *Drosophila*, we performed PCR with fly head cDNA and the primers made from conserved amino acids in TMs VI and VII of a *Drosophila* tyramine–octopamine receptor (Arakawa et al., 1990; Saudou et al., 1990). Known biogenic amine receptors are relatively uniform in length between TMs VI and VII, which minimizes the differential amplification of receptor subsets attributable to heterogeneity in length between the PCR primers. Because of this, however, distinguishing novel PCR products from known products on the basis of size becomes problematic. To circumvent this, we used SSCP (Orita et al., 1989) to screen the PCR products. The PCR products were denatured to generate single strands and then subjected to nondenaturing gel electrophoresis. Denatured single-stranded DNA forms some secondary structure based on primary sequence, allowing for resolution of DNA fragments by length and conformation.

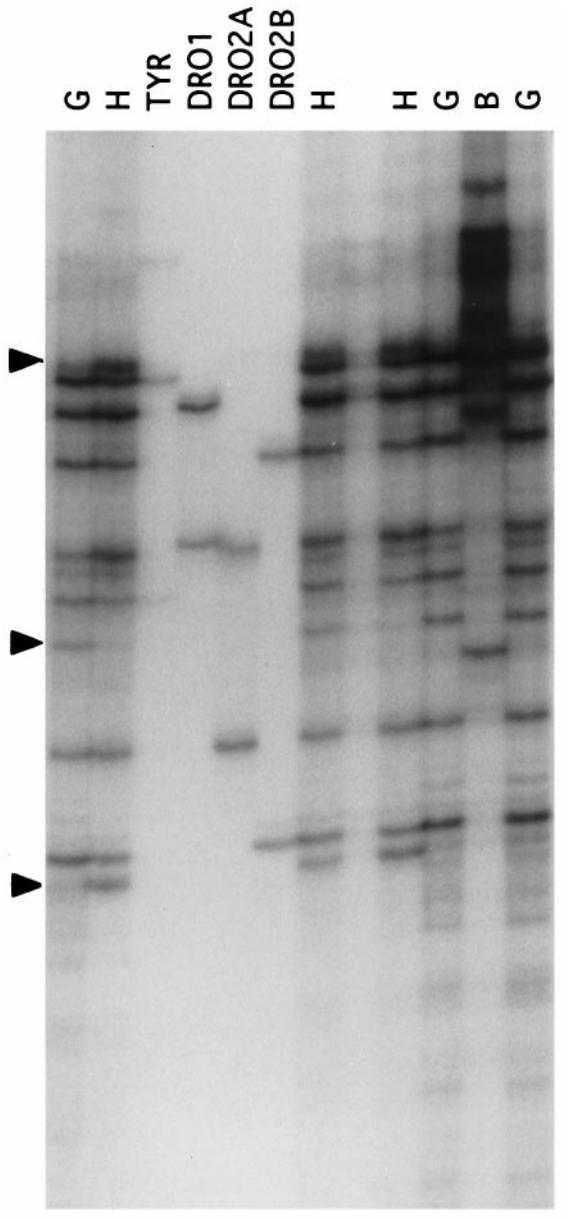

Figure 1. PCR and SSCP. PCR products were generated from fly head cDNA (*H*), body cDNA (*B*), or genomic DNA (*G*) and resolved on a 4% nondenaturing gel. The cDNAs for the tyramine receptor (*TYR*) and three different serotonin receptors (*DRO1*, *DRO2A*, and *DRO2B*) were used for counterscreening. The *arrowheads* mark some of the unique PCR products identified with head cDNA.

The resolution and sensitivity of SSCP were first tested using PCR products made from head and body cDNA as well as genomic DNA (Fig. 1). Independent PCRs using the same tem-

plates were loaded on the gel to eliminate PCR artifacts and to align the bands in the various lanes. Approximately a dozen strongly labeled bands were observed using head cDNA as a template (Fig. 1). The spectrum of bands observed with body cDNA, however, was quite distinct. This suggested that the predominant receptor types found in the body are generally nonoverlapping with the predominant receptors found in the head. The PCR products from genomic DNA would be expected to be the sum of those produced from head and body cDNA. Although this was observed, the bands representing body receptor products from genomic DNA were very weak, and most major products were identical to those using head cDNA as a template. The primers used for PCR were not degenerate but represented the sequences of the TYR. Thus, receptor RNAs with the highest sequence identity would be amplified preferentially. It seems possible that head cDNA contains receptor sequences more similar to the primers used than does the body cDNA.

The cDNA clones representing four known biogenic amine receptors identified from *Drosophila* were also used in the analysis. These included one TYR (Saudou et al., 1990) and three serotonin receptors (DRO1, DRO2A, and DRO2B) (Witz et al., 1990; Saudou et al., 1992). These known receptors produced PCR products that co-migrated with approximately two-thirds of the major PCR products made from head cDNA (Fig. 1). This indicated that several unidentified receptor RNAs exist in the head RNA population with high sequence similarity to the primers. To identify these RNAs and others that might not be resolved in the gel, the complete population of PCR products amplified from head cDNA was cloned. Approximately 100 independent clones were screened by SSCP for unique mobilities against the four known receptors, yielding four clones representing novel receptor sequences, as revealed after isolation of the corresponding cDNAs and sequencing. One of these is a novel D1 dopamine receptor, DAMB (Han et al., 1996); a second, OAMB, is described here.

OAMB sequence predicts a biogenic amine receptor

A 114 bp clone obtained by PCR was used to screen a genomic DNA library. A fragment from one positive phage was, in turn, used to screen a head cDNA library. The subsequently identified cDNA clone (OAMB) of 3387 bp contained a methionine followed by a long open reading frame predicting a protein of 637 amino acids (Fig. 2*A*). Hydropathy profiles (data not shown) revealed seven hydrophobic domains with striking similarities to the TMs of GPRs (Vernier et al., 1993). An Asp residue was found in TM III, and two Ser residues were found in TM V (Fig. 2*A*). These residues constitute part of the binding site for biogenic amines of other receptors (Vernier et al., 1993), implying that OAMB belongs to the biogenic amine receptor superfamily. This putative receptor clone also contained two consensus sites for N-linked glycosylation (N-*X*-S/T) in the putative extracellular N terminus and the second extracellular loop. Ten consensus phosphorylation sequences for protein kinase C [(R/K) . . . S/T-*X*-R/K], two for protein kinase A (K/R-R-*X*-T/S), and five for calcium–calmodulin-dependent protein kinase II (R-*X*-Y-S/T) were found in the cytoplasmic domains (Fig. 2*A*). These sites might be involved in dynamic regulation of receptor activity. A unique feature of OAMB is its long extracellular loop (130 amino acids) between TM IV and V. Most biogenic amine receptors contain a relatively short loop ranging from 10 to 30 amino acids. Although no recognizable motif was found in this extended sequence, it is attempting to speculate its function for interaction with cell adhesion molecules or extracellular matrix for regulation of receptor activity.

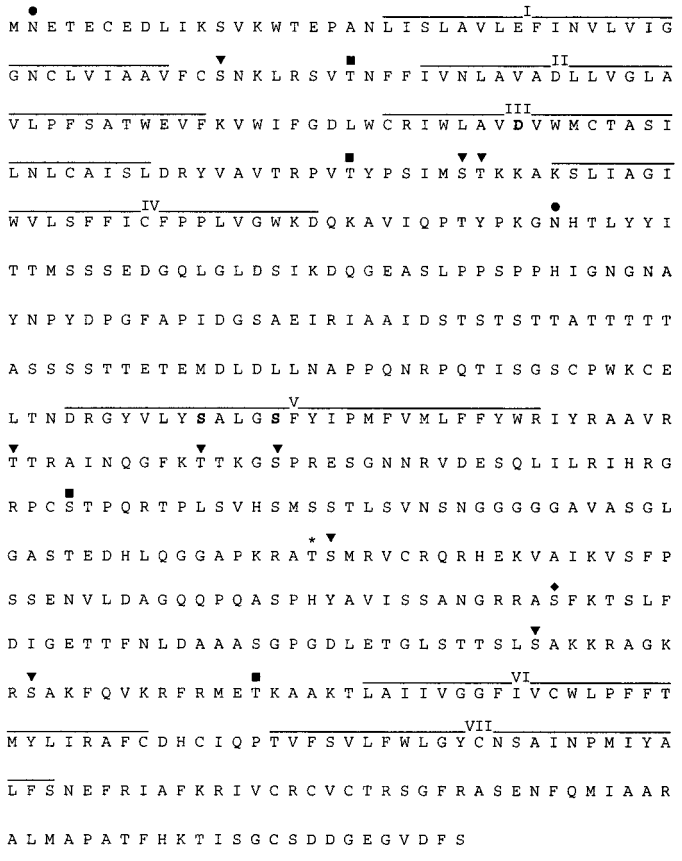

Figure 2. Deduced amino acid sequence of OAMB and alignment of OAMB with other biogenic amine receptors. *A*, The seven putative TMs are indicated by *overlining* and *roman numerals*. *Circles*, *asterisk*, *triangles*, and *squares* mark putative N-linked glycosylation sites, protein kinase A, protein kinase C, and calcium–calmodulin-dependent protein kinase II phosphorylation sites, respectively. The serine (*S*) marked as a *diamond* is a putative phosphorylation site by all kinases indicated above. The Asp residue (*D*) in TM III and the two Ser residues (*S*) in TM V thought to interact with octopamine are indicated in *bold*. OAMB has been mapped to the cytological location 92F. *B*, The deduced amino acid sequence of OAMB is aligned with the barnacle GPR (*GPR-BAR*), the *Drosophila* tyramine receptor (*TYRR-DRO*), the human α1 adrenergic receptor (*A1AB-HUM*), the α2 receptor (*A2AA-HUM*), the β1 receptor (*B1AR-HUM*), and the *Drosophila* dopamine DAMB receptor (*DAMB-DRO*). Predicted TMs I–VII are *overlined*. *Numbers* in *parentheses* correspond to the number of amino acids at the N and C termini and in the second and the third cytoplasmic loops that are not represented in the figure. The amino acids that are conserved in all compared receptors are *shaded*.

Comparison of the predicted amino acid sequence of OAMB with protein data banks revealed the highest sequence identity (39%) with a barnacle GPR with unknown ligand specificity (Isoai et al., 1996). Other biogenic amine receptors displayed similar overall identity (25–30%) with OAMB, confined primarily to the seven TMs. When the TMs were compared (Fig. 2*B*), the degree of sequence identity increased to 72% for the barnacle GPR; 52–55% for human α1 adrenergic receptors (Ramarao et al., 1992), the *Drosophila* DAMB (Han et al., 1996), and the TYR (Saudou et al., 1990); and 45–50% for human α2 and β adrenergic receptors (Frielle et al., 1987; Fraser et al., 1989). The failure to find a high identity to any one receptor subfamily suggests that OAMB represents the prototypic member of a new receptor subfamily that may include the barnacle GPR.

```
                 ____________I____________                 _____________II____________
OAMB-DRO   ( 20)-LISLAVLEFINVLVIGGNCLVIAAVFCSNKLRSVTNFFIVNLAVADLLVGLAVLPFSATWEVFK-VWIFGDLW
GPR -BAR   ( 33)-LISLLALAFLNLMVVAGNLLVVMAVFVHSKLRTVTNLFIVSLACADLLVGMLVLPFSATLEVLD-VWLYGDVW
TYRR-DRO   (109)-LLTALVLSVIIVLTIIGNILVILSVFTYKPLRIVQNFFIVSLAVADLTVALLVLPFNVAYSILG-RWEFGIHL
A1AB-HUM   ( 45)-ISVGLVLGAFILFAIVGNILVILSVACNRHLRTPTNYFIVNLAMADLLLSFTVLPFSAALEVLG-YWVLGRIF
A2AA-HUM   ( 33)-LTLVCLAGLLMLLTVFGNVLVIIAVFTSRALKAPQNLFLVSLASADILVATLVIPFSLANEVMG-YWYFGKAW
B1AR-HUM   ( 58)-AGMGLLMALIVLLIVAGNVLVIVAIAKTPRLQTLTNLFIMSLASADLVMGLLVVPFGATIVVWG-RWEYGSFF
DAMB-DRO   (108)-VGLLAFLFLFSFATVFGNSLVILAVIRERYLHTATNYFITSLAVADCLVGLVVMPFSALYEVLENTWFFGTDW

           ___________III____________                 _____________IV____________
OAMB-DRO   CRIWLAVDVWMCTASILNLCAISLDRYVAVTRPVTYPSIMSTKKAKSLIAGIWVLSFFICFPPLVGWKDQKAVIQPTYP
GPR -BAR   CSVWLAVDVWMCTSSILNLCAISLDRYLAVSQPISYPSLMSTRRAKQLIAAVWVLSFVICFPPLVGWNDRPGTLIGSRG
TYRR-DRO   CKLWLTCDVLCCTSSILNLCAIALDRYWAITDPINYAQKRTVGRVLLLISGVWLLSLLISSPPLIGWNDWPDEFTSATP
A1AB-HUM   CDIWAAVDVLCCTASILSLCAISIDRYIGVRYSLQYPTLVTRRKAILALLSVWVLSTVISIGPLLGWKEPAPNDDKE--
A2AA-HUM   CEIYLALDVLFCTSSIVHLCAISLDRYWSITQAIEYNLKRTPRRIKAIIITVWVISAVISFPPLISIEKKGGGGGPQPA
B1AR-HUM   CELWTSVDVLCVTASIETLCVIALDRYLAITSPFRYQSLLTRARARGLVCTVWAISALVSFLPILMHWWRAESDEARRC
DAMB-DRO   CDIWRSLDVLFSTASILNLCVISLDRYWAITDPFSYPMRMTVKRAAGLIAAVWICSSAISFPAIVWWRAARDGEMPAYK

                           _____________V_____________                              _____
OAMB-DRO   (115)--CELTNDRGYVLYSALGSFYIPMFVMLFFYWRIYRAAVRTTRAIN-(177)-KFQVKRFRMETKAAKTLAIIV
GPR -BAR   SSACRLTCELTNERGYVIYSALGSFFLPSTVMLFFYGRIYRTAVSTTRAIA-(124)-RHQARRFRMETKAAKTVGIIV
TYRR-DRO   -------CELTSQRGYVIYSSLGSFFIPLAIMTIVYIEIFVATRRRLRERA-(210)-EKQKISLSKERRAARTLGIIM
A1AB-HUM   -------CGVTEEPFYALFSSLGSFYIPLAVILVMYCRVYIVAKRTTKNLE-( 41)-AVKLFKFSREKKAAKTLGIVV
A2AA-HUM   EPR----CEINDQKWYVISSCIGSFFAPCLIMILVYVRIYQIAKRRTRVPP-(128)-RAGAGGQNREKRFTFVLAVVI
B1AR-HUM   YNDPKC-CDFVTNRAYAIASSVVSFYVPLCIMAFVYLRVFREAQKQVKKID-( 50)-RPSRLVALREQKALKTLGIIM
DAMB-DRO   -------CTFTEHLGYLVFSSTISFYLPLLVMVFTYCRIYRAAVIQTRSLK-(100)-SRKLAKFAKEKKAAKTLGIVM

           _________VI_____________          _____________VII____________
OAMB-DRO   GGFIVCWLPFFTMYLIRAFCDHCIQP--TVFSVLFWLGYCNSAINPMIYALFSNEFRIAFKRIVCRCVCTRSGF-( 37)
GPR -BAR   GLFILCWLPFFVCYLVRGFCADCV-PP-LLFSVFFWLGYCNSAVNPCVYALCSRDFRFAFSSILCKCVCRRGAM-( 24)
TYRR-DRO   GVFVICWLPFFLMYVILPFCQTCC-PTNKFKNFITWLGYINSGLNPVIYTIFNLDYRRAFKRLLGLN
A1AB-HUM   GMFILCWLPFFIALPLGSLFSTLK-PPDAVFKVVFWLGYFNSCLNPIIYPCSSKEFKRAFVRILGCQCRGRRRR-(144)
A2AA-HUM   GVFVVCWFPFFFTYTLTAVGCS--VP-RTLFKFFFWFGYCNSSLNPVIYTIFNHDFRRAFKKILCRGDRKRIV
B1AR-HUM   GVFTLCWLPFFLANVVKAFHRE-LVP-DRLFVFFNWLGYANSAFNPIIY-CRSPDFRKAFQGLLCCARRAARRR-( 76)
DAMB-DRO   GVFIICWLPFFVVNLLSGFCIECIEHEEIVSAIVTWLGWINSCMNPVIYACWSRDFRRAFVRLLCMCCPRKIRR-( 38)
```

Figure 2 continued.

Octopamine-induced cAMP and $[Ca^{2+}]_i$ increase through OAMB

To investigate the functional properties of OAMB, a stable cell line was established by transfection of *Drosophila* S2 cells with the OAMB cDNA and assayed for intracellular cAMP accumulation in the presence of various neuromodulators (Han et al., 1996). No significant changes in cAMP levels were detected in either transfected (S2-OAMB) or untransfected S2 cells treated with serotonin, dopamine, or histamine (up to 10 μM; data not shown). In contrast, octopamine at 10 μM induced a marked increase in cAMP levels ~10-fold in transfected but not in untransfected cells. Tyramine and norepinephrine at 10 μM also produced smaller but significant elevations in cAMP levels only in transfected cells. Dose–response curves for octopamine, tyramine, and norepinephrine (Fig. 3*A*) indicated that the octopamine-induced cAMP increase was concentration-dependent and saturable, with an EC_{50} of $1.9 \pm 0.5 \times 10^{-7}$ M. Tyramine and norepinephrine were ~100-fold less potent than octopamine and generated ~70–80% of the maximal response seen with octopamine (Fig. 3*A*).

We also examined the functional properties of OAMB in transiently transfected human embryonic kidney (HEK-OAMB) cells. As shown for the S2-OAMB cells, octopamine and, to a lesser extent, tyramine induced a marked elevation in cAMP in transfected cells but had no effect in untransfected HEK cells (data not shown). The EC_{50} for both octopamine and tyramine determined in HEK-OAMB cells was essentially indistinguishable from the values determined in S2-OAMB cells. Together, these results indicate that OAMB represents a functional octopamine receptor that can couple positively with AC to stimulate cAMP production in both *Drosophila* and human cells.

The ability of OAMB to alter $[Ca^{2+}]_i$ was also examined in HEK cells expressing OAMB. After loading cells with the Ca^{2+}-sensitive dye fura-2 AM, a clear increase in $[Ca^{2+}]_i$ was observed in response to octopamine at 10^{-5} M but not to dopamine (Fig. 3*B*). No response was detected in nontransfected cells (data not shown) or in cells expressing the DAMB receptor. The latter cells did show, however, an increase in $[Ca^{2+}]_i$ in response to dopamine but not to octopamine. These data indicate that the

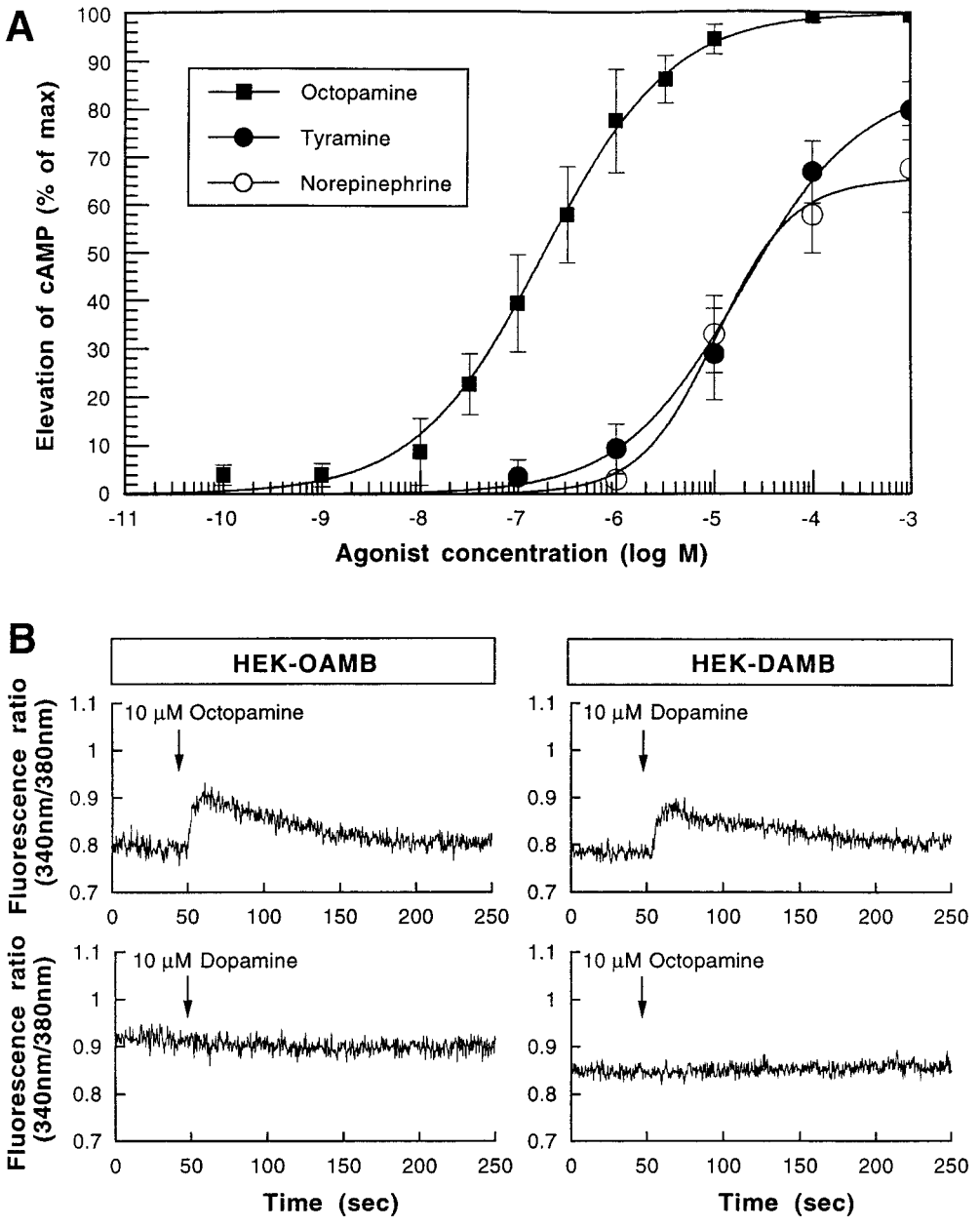

Figure 3. Agonist modulation of cAMP and $[Ca^{2+}]_i$ levels in *Drosophila* S2 cells and human HEK cells. *A*, Dose–response curves for agonist-induced elevation in cAMP levels in the S2-OAMB cells. All data points are the means of three independent determinations and have been normalized to the maximum response to octopamine. The curves were fitted by least squares method. The mean calculated EC_{50} value for octopamine is 0.19 ± 0.05 μM. *B*, Agonist-induced elevation of $[Ca^{2+}]_i$ in transiently transfected *HEK-OAMB* or *HEK-DAMB* cells loaded with fura2-AM. Agonists were applied at 10 μM. Increase in $[Ca^{2+}]_i$ is represented by the measured fluorescence ratio (340: 380 nm).

increase in $[Ca^{2+}]_i$ by octopamine in OAMB-expressing cells is mediated by OAMB. Thus, activation of OAMB stimulated intracellular accumulations of cAMP and Ca^{2+} in transfected HEK cells.

The OAMB receptor is preferentially expressed in mushroom bodies

To examine the tissue distribution of OAMB, RNA blots of head and body fractions were probed with an OAMB cDNA clone. Two major mRNA species of 4.2 and 3.5 kb were detected in the head fraction but not in the body fraction (Fig. 4), indicating that the OAMB transcripts were specific to fly heads. *In situ* hybridization was performed to determine cell types that express OAMB. A series of frontal head sections was hybridized with digoxigenin-labeled riboprobes representing the 5′ half of the OAMB cDNA (Fig. 5). The OAMB transcripts, detected with antisense (Figs. 5*A*) but not sense (Fig. 5*D*) RNA probes, were present preferentially in perikarya of mushroom bodies situated in the dorsal and posterior brain cortex. However, OAMB expression was not uniform among all mushroom body neurons; some mushroom body neurons exhibited intense signals, whereas others stained at relatively lower levels (Fig. 5*C*). Similarly, two clusters of cells located in the anterior brain cortex near the mushroom body lobes stained intensely for OAMB transcripts (Fig. 5*B*). A relatively low signal was observed in cells scattered in the central brain (Fig. 5*A*) and medulla of the optic lobes (data not shown). No significant signal was detectable in other tissues, including muscle (Fig. 5*A*) and fat cells (data not shown).

To determine the distribution of the OAMB receptor within the mushroom bodies and other brain structures, head sections were stained with a polyclonal antibody generated against the third cytoplasmic loop of the receptor. Strong immunoreactivity was observed in the neuropil that house the mushroom body dendrites (calyces; Fig. 5*E*), axons (pedunculi; Fig. 5*G*,*H*), and axon terminals (α, β, and γ lobes; Figs. 5*F-H*). Distinct immuno-

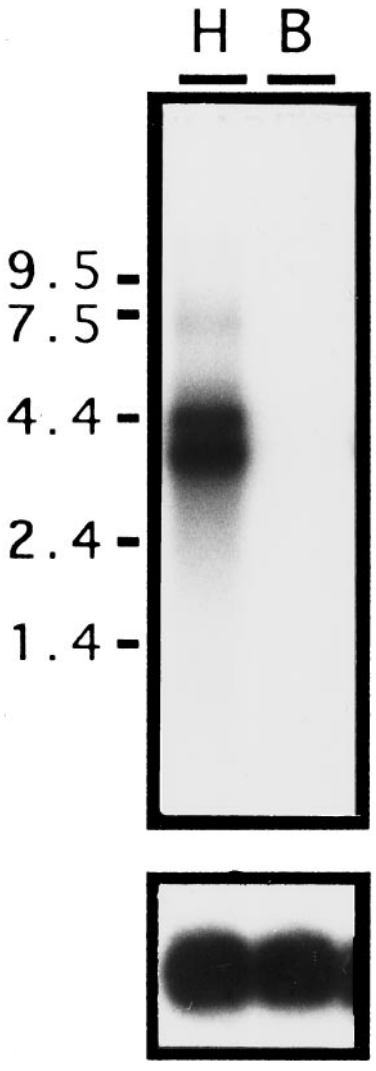

Figure 4. RNA blotting of OAMB. Ten micrograms of poly(A^+) RNA from heads (*H*) or bodies (*B*) were resolved by gel electrophoresis, transferred to a nylon membrane, and hybridized with a ^{32}P-labeled OAMB cDNA clone (*top*) or a ribosomal protein rp49 cDNA clone (*bottom*) (O'Connell and Rosbash, 1984) as a loading control. Molecular size markers (in kilobases) are indicated.

reactivity was also detected in ellipsoid body of central complex (Fig. 5*G*). This observation suggests that some cells in the anterior brain cortex that hybridized to antisense OAMB (Fig. 5*B*) may represent neurons projecting to the ellipsoid body. No specific immunoreactivity was observed in other regions of heads or bodies. We conclude that the novel octopamine receptor OAMB is highly enriched in the mushroom body and ellipsoid body neuropils.

DISCUSSION

In the present study, we have identified a novel octopamine receptor that stimulates both cAMP and $[Ca^{2+}]_i$ accumulation. The PCR–SSCP approach used to isolate the receptor is unique and advantageous. SSCP analysis relies on the electrophoretic mobility of single-stranded DNA conferred primarily by secondary structure, detecting even single-nucleotide alterations. Therefore, a parallel comparison of PCR products from various tissue samples along with known sequences allows for a rapid way of identifying novel sequences of low abundance among closely related genes. We obtained four novel GPR clones from fly heads using this approach (K.-A. Han and R. L. Davis, unpublished observations), and further analysis identified one of them as a novel dopamine receptor DAMB and another as an octopamine receptor OAMB. Although we have restricted our analysis by screening a small fraction of the recombinant colonies (<0.01%), it should be possible to perform a near-saturation screen for a family of genes by designing various combinations of primer sets and screening recombinant colonies on a larger scale by colony hybridization and sequencing.

Although octopamine is a principal neuromediator in insects, its receptors have proven difficult to clone. Pharmacological profiles of octopamine receptors are well characterized in locust. One neuronal and three non-neuronal receptor subtypes with distinct affinity for ligands and different effectors have been found (Evans and Robb, 1993; Roeder, 1995). One octopamine-sensitive receptor has been cloned from *Drosophila* heads, but tyramine is two orders of magnitude more potent than octopamine in inhibiting AC activity through this receptor. However, both ligands show similar potencies for increasing $[Ca^{2+}]_i$. Thus, it has been classified as a tyramine–octopamine receptor (Saudou et al., 1990; Robb et al., 1994). Octopamine binds with high affinity at sites distinct from tyramine in membranes prepared from fly heads and is a potent activator of AC (Uzzan and Dudai, 1982; Dudai and Zvi, 1984). The OAMB receptor identified here satisfies such observations, because OAMB stimulated AC with higher efficacy to octopamine over tyramine in both mammalian and *Drosophila* cell lines. In addition, OAMB mediated a $[Ca^{2+}]_i$ increase in response to octopamine. It is possible that the $[Ca^{2+}]_i$ increase results from the activation of enzymes such as phospholipase C. A recently cloned octopamine receptor from the pond snail *Lymnaea* is coupled to both AC and phospholipase C (Gerhardt et al., 1997).

Several lines of evidence indicate that octopamine plays essential roles in behavioral plasticity in *Drosophila*. Although no information is available on the performance of flies with reduced octopamine content (*inactive*) and without octopamine (*tβh*) in classical olfactory conditioning, flies fed with formamidines display impaired olfactory learning, although they can sense the relevant stimuli presented during training (Dudai et al., 1987). These drugs not only interact with octopamine binding sites but also exhibit antagonistic effects on octopamine-induced cAMP production. This suggests that an octopamine receptor positively coupled to AC mediates olfactory learning. In addition, *inactive* mutant males display impaired experience-dependent courtship modification toward mature males (O'Dell, 1994). The necessity for functional octopamine binding sites and cAMP metabolism for normal learning suggests that OAMB may serve as a receptor for octopaminergic input to mediate synaptic plasticity. Intriguingly, OAMB is highly enriched in the mushroom body neuropil, a principal neuroanatomical site mediating normal olfactory conditioning and conditioned courtship (O'Dell et al., 1995; Davis, 1996; Davis and Han, 1996). These observations suggest that OAMB may modulate the physiology of mushroom bodies underlying associative learning. However, multiple biogenic amine receptors are likely to be important for learning, because we have previously shown that the novel dopamine receptor DAMB is localized preferentially to the mushroom body lobes and pedunculi (Han et al., 1996).

Therefore, the anatomical and biochemical architecture may be arranged such that conditioned stimulus (CS) and unconditioned

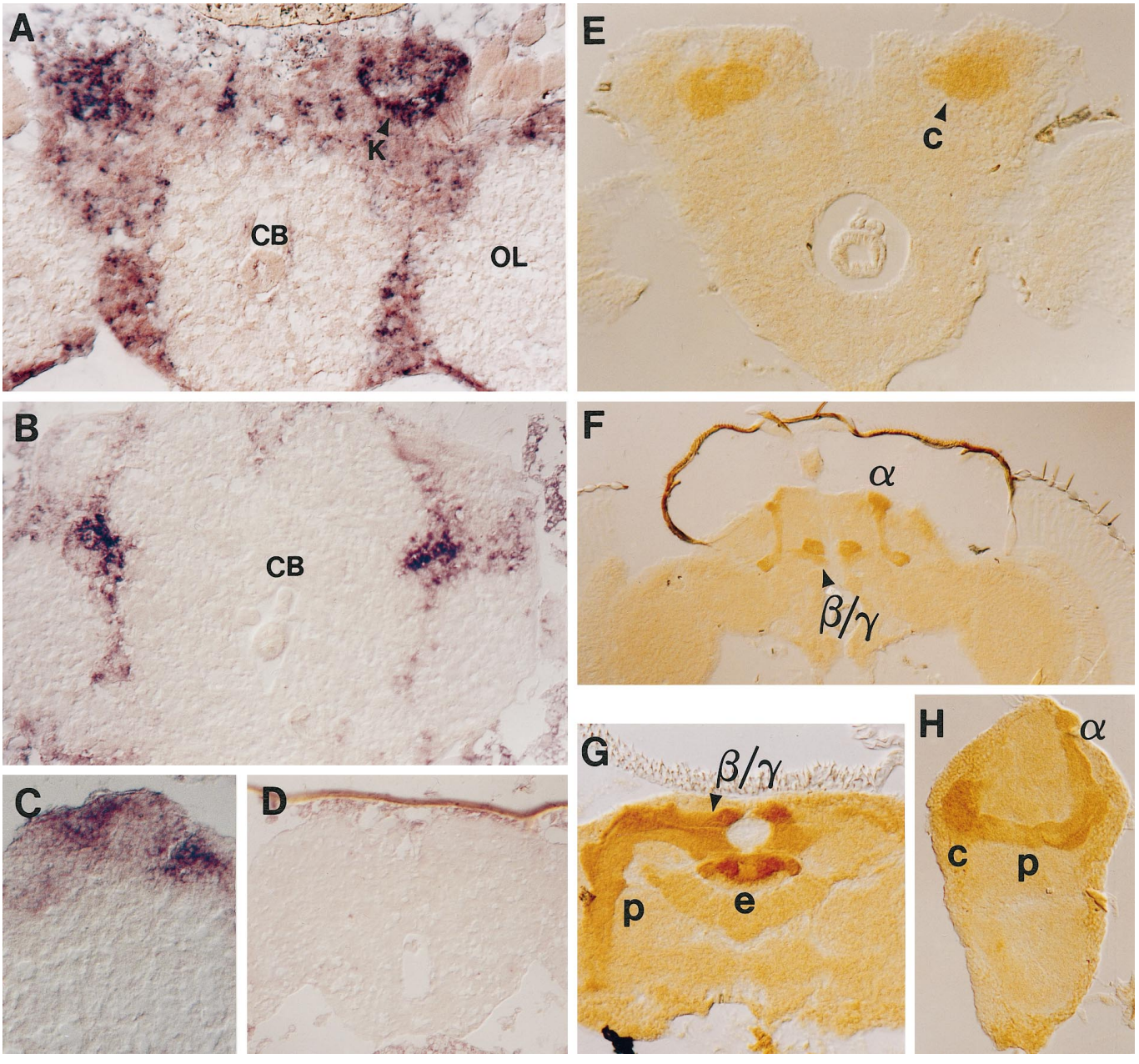

Figure 5. Expression of OAMB in mushroom bodies. *A–D*, *In situ* hybridization. *A*, Frontal section of the posterior brain. Central brain (*CB*) and optic lobe (*OL*) areas are indicated. *K*, Kenyon (mushroom body) cells. *B*, Frontal section of the anterior brain. *C*, *D*, Frontal section at the level of the calyces. *A–C* were hybridized with an antisense OAMB probe, and *D* was hybridized with a sense OAMB probe. *E–H*, Immunohistochemistry. *E*, Frontal section at the level of calyces. *F*, Frontal section at the level of lobes. α, α lobes; β, β lobes; γ, γ lobes. *G*, Horizontal section. *e*, Ellipsoid body; *p*, pedunculus. *H*, Sagittal section. *c*, Calyx. The head sections were incubated with anti-OAMB antibody. For all frontal sections, dorsal is *top*. Anterior is *top* in *G* and to the *right* in *H*. Magnification: *D, F*, 200×; *A–C, E, G, H*, 400×.

stimulus (US) inputs converge at the mushroom bodies through distinct receptors and activate signal transduction cascades to modulate the synaptic output of mushroom bodies (Fig. 6). Odor information received during olfactory conditioning is conveyed to the mushroom body calyces (Strausfeld, 1976), presumably through cholinergic transmission (Restifo and White, 1990), and encoded as a CS, perhaps in part through modulatory actions of the octopamine receptor OAMB. This receptor may be activated by modulatory neurons delivering US information to trigger cAMP accumulation through AC or $[Ca^{2+}]_i$ increase through phospholipase C. How odors are encoded and how simultaneous US input might convert an odor into a CS are unknown, although studies in larger insects (Laurent and Davidowitz, 1994) implicate synchronous oscillation in the antennal lobes or the mushroom bodies as part of the physiological basis for odor recognition. It is possible that OAMB may modify this process to convert odor information into a CS. Because formamidine treatment significantly reduces the learning of *rut* flies (Dudai et al., 1987), other ACs (Levine et al., 1992) distinct from the *rut* AC may be coupled to OAMB. Alternatively, OAMB may use $[Ca^{2+}]_i$ to mediate the US information. That octopamine mediates US information has been demonstrated using the proboscis extension reflex conditioning of the honeybee, in which octopamine injection into the calyces during training substitutes for a sugar reward given to the

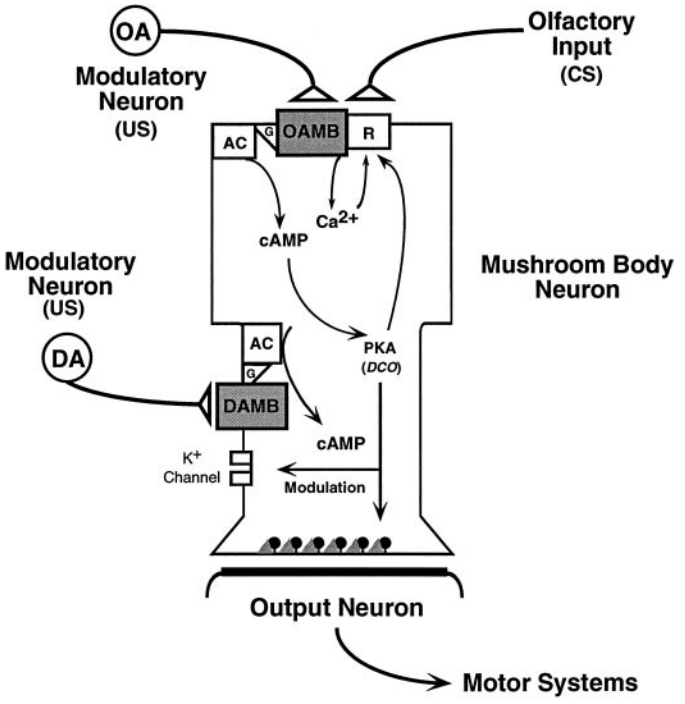

Figure 6. Model for the cAMP cascade triggered by OAMB during olfactory conditioning. Olfactory information (*CS*) is received at the antennae and conveyed to the dendrites of mushroom body cells via the antennoglomerular tract. The olfactory information presented to the mushroom body cells may be modified from activation of OAMB by concurrent *US* inputs from modulatory neurons. Increases in $[Ca^{2+}]_i$ or cAMP from activation of OAMB may be responsible for modifying or processing the *CS* information. Information representing the *US* may also be mediated by dopaminergic (*DA*) modulatory neurons that activate the dopamine receptor (*DAMB*) situated on the pedunculi and lobes. Activated *DAMB* stimulates *AC*, leading to increased cAMP production. The elevated cAMP then modulates the synaptic output of mushroom body neurons to motor circuits, either directly through cyclic nucleotide-sensitive potassium channels or indirectly through protein kinase A (*PKA*), which in turn phosphorylates ion channels and other molecules to encode memory (Davis, 1996).

proboscis (Menzel et al., 1996). Given the diversity of neuromodulatory systems, it is also conceivable that OAMB may process different sensory inputs. The newly identified receptors DAMB and OAMB with distinct biochemical and anatomical properties should help dissect the elaborate neuromodulatory systems underlying behavioral plasticity.

REFERENCES

Arakawa S, Gocayne JD, McCombie WR, Urquhar TDA, Hall LM, Fraser CM, Venter JC (1990) Cloning localization and permanent expression of a *Drosophila* octopamine receptor. Neuron 4:343–354.

Braun G, Bicker G (1992) Habituation of an appetitive reflex in the honeybee. J Neurophysiol 67:588–598.

Connolly JB, Roberts IJ, Armstrong JD, Kaiser K, Forte M, Tully T, O'Kane CJ (1996) Associative learning disrupted by impaired Gs signaling in *Drosophila*: mushroom bodies. Science 274:2104–2107.

Cooper ST, Millar NS (1997) Host cell-specific folding and assembly of the neuronal nicotinic acetylcholine receptor alpha 7 subunit. J Neurochem 68:2140–2151.

David J-C, Coulon J-F (1985) Octopamine in invertebrates and vertebrates. A review. Prog Neurobiol 24:141–185.

Davis RL (1996) Physiology and biochemistry of *Drosophila* learning mutants. Physiol Rev 76:299–317.

Davis RL, Han K-A (1996) Mushrooming mushroom bodies. Curr Biol 6:146–148.

de Belle JS, Heisenberg M (1994) Associative odor learning in *Drosophila* abolished by chemical ablation of mushroom bodies. Science 263:692–695.

Dudai Y, Zvi S (1984) High-affinity [^{3}H]octopamine-binding sites in *Drosophila melanogaster*: interaction with ligands and relationship to octopamine receptors. Comp Biochem Physiol C Comp Pharmacol Toxicol Endocrinol 77:145–151.

Dudai Y, Buxbaum J, Corfas G, Ofarim M (1987) Formamidines interact with *Drosophila* octopamine receptors alter the flies' behavior and reduce their learning ability. J Comp Physiol [A] 161:739–746.

Evans PD, Robb S (1993) Octopamine receptor subtypes and their modes of action. Neurochem Res 18:869–874.

Forte M, Quan F, Hyde D, Wolfgang W (1993) Gα proteins in *Drosophila*: structure and developmental expression. In: GTPase in biology (Dickey B, Birnbaumer L, eds), pp 319–334. Heidelberg: Springer.

Fraser CM, Arakawa S, McCombie WR, Venter JC (1989) Cloning sequence analysis and permanent expression of a human alpha 2-adrenergic receptor in Chinese hamster ovary cells. Evidence for independent pathways of receptor coupling to adenylate cyclase attenuation and activation. J Biol Chem 264:11754–11761.

Frielle T, Collins S, Daniel KW, Caron MG, Lefkowitz RJ, Kobilka BK (1987) Cloning of the cDNA for the human beta 1-adrenergic receptor. Proc Natl Acad Sci USA 84:7920–7924.

Gerhardt CC, Bakker RA, Piek GJ, Planta RJ, Vreugdenhil E, Leysen JE, Van Heerikhuizen H (1997) Molecular cloning and pharmacological characterization of a molluscan octopamine receptor. Mol Pharmacol 51:293–300.

Glanzman DL, Kranse FB (1983) Serotonin and octopamine have opposite modulatory effects on the crayfish's lateral giant escape reaction. J Neurosci 3:2263–2269.

Grotewiel MS, Beck CDO, Wu KH, Zhu X-R, Davis RL (1998) Integrin-mediated short-term memory in *Drosophila*. Nature, 391:455–460.

Han K-A, Kulesz-Martin MF (1992) Altered expression of wild-type p53 tumor suppressor gene during murine epithelial cell transformation. Cancer Res 52:749–753.

Han K-A, Millar NS, Grotewiel MS, Davis RL (1996) DAMB, a novel dopamine receptor expressed specifically in *Drosophila* mushroom bodies. Neuron 16:1127–1135.

Han P-L, Levin LR, Reed RR, Davis RL (1992) Preferential expression of the *Drosophila rutabaga* gene in mushroom bodies, neural centers for learning in insects. Neuron 9:619–627.

Heisenberg M, Borst A, Wagner S, Byers D (1985) *Drosophila* mushroom body mutants are deficient in olfactory learning. J Neurogenet 2:1–30.

Isoai A, Kawahara HYO, Shizuri Y (1996) Molecular cloning of a new member of the putative G protein-coupled receptor gene from barnacle, *Balanus amphitrite*. Gene 175:95–100.

Laurent G, Davidowitz H (1994) Encoding of olfactory information with oscillating neural assemblies. Science 265:1872–1875.

Levin LR, Han PL, Hwang PM, Feinstein PG, Davis RL, Reed RR (1992) The *Drosophila* learning and memory gene *rutabaga* encodes a Ca^{2+}/calmodulin-responsive adenylyl cyclase. Cell 68:479–489.

Livingstone M, Harris-Warrick RM, Kravitz EA (1980) Serotonin and octopamine produce opposite postures in lobsters. Science 208:76–79.

Long TF, Edgecomb RS, Murdock LL (1986) Effects of substituted phenylethylamines on blowfly feeding behavior. Comp Biochem Physiol C Comp Pharmacol Toxicol Endocrinol 83:201–209.

Menzel R, Hammer M, Muller U, Rosenboom H (1996) Behavioral neural and cellular components underlying olfactory learning in the honeybee. J Physiol (Paris) 90:395–398.

Millar NS, Buckingham SD, Sattelle DB (1994) Stable expression of a functional homo-oligomeric *Drosophila* GABA receptor in a *Drosophila* cell line. Proc R Soc Lond B Biol Sci 258:307–314.

Monastirioti M, Gorczyca M, Rapus J, Eckert M, White K, Budnik V (1995) Octopamine immunoreactivity in the fruit fly *Drosophila melanogaster*. J Comp Neurol 356:275–287.

Monastirioti M, Linn JCE, White K (1996) Characterization of *Drosophila* tyramine b-hydroxylase gene and isolation of mutant flies lacking octopamine. J Neurosci 16:3900–3911.

Nighorn A, Healy MJ, Davis RL (1991) The cyclic AMP phosphodiesterase encoded by the *Drosophila dunce* gene is concentrated in the mushroom body neuropil. Neuron 6:455–467.

O'Connell PO, Rosbash M (1984) Sequence structure and codon preference of the *Drosophila* ribosomal protein 49 gene. Nucleic Acids Res 12:5495–5513.

O'Dell KM (1993) The effect of the inactive mutation on longevity sex rhythm and resistance to *p*-cresol in *Drosophila melanogaster*. Heredity 70:393–399.

O'Dell KM (1994) The inactive mutation leads to abnormal experience-dependent courtship modification in male *Drosophila melanogaster*. Behav Genet 24:381–388.

O'Dell KMC, Armstrong JD, Yang MY, Kaiser K (1995) Functional dissection of the *Drosophila* mushroom bodies by selective feminization of genetically defined subcompartments. Neuron 15:55–61.

Orita M, Suzuki Y, Sekiya T, Hayashi K (1989) Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. Genomics 5:874–879.

Ramarao CS, Denker JM, Perez DM, Gaivin RJ, Riek P, Graham RM (1992) Genomic organization and expression of the human alpha 1B-adrenergic receptor. J Biol Chem 267:21936–21945.

Restifo LL, White K (1990) Molecular and genetic approaches to neurotransmitter and neuromodulator systems in *Drosophila*. Adv Insect Physiol 22:115–219.

Robb S, Cheek TR, Hannan FL, Hall LM, Midgley JM, Evans PD (1994) Agonist-specific coupling of a cloned *Drosophila* octopamine/tyramine receptor to multiple second messenger systems. EMBO J 13:1325–1330.

Roeder T, Degen J, Dyczkowski C, Gewecke M (1995) Pharmacology and molecular biology of octopamine receptors from different insect species. Prog Brain Res 106:249–258.

Saudou F, Amlaiky N, Plassat JL, Borrelli E, Hen R (1990) Cloning and characterization of a *Drosophila* tyramine receptor. EMBO J 9:3611–3617.

Saudou F, Boschert U, Amlaiky N, Plassat JL, Hen R (1992) A family of *Drosophila* serotonin receptors with distinct intracellular signaling properties and expression patterns. EMBO J 11:7–17.

Schürmann F-W (1987) The architecture of the mushroom bodies and related neuropils in the insect brain. In: Arthropod Brain (Gupta AP, ed), pp 231–264. New York: Wiley.

Skoulakis EM, Davis RL (1996) Olfactory learning deficits in mutants for *leonardo*, a *Drosophila* gene encoding a 14–3-3 protein. Neuron 17:931–944.

Skoulakis EM, Kalderon D, Davis RL (1993) Preferential expression in mushroom bodies of the catalytic subunit of protein kinase A and its role in learning and memory. Neuron 11:1–14.

Strausfeld N (1976) Atlas of an insect brain. Heidelberg: Springer.

Uzzan A, Dudai Y (1982) Aminergic receptors in *Drosophila melanogaster*: responsiveness of adenylate cyclase to putative neurotransmitters. J Neurochem 38:1542–1550.

Vernier P, Philippe H, Samama P, Mallet J (1993) Bioamine receptors: evolutionary and functional variations of a structural leitmotiv. EXS 63:297–337.

Witz P, Amlaiky N, Plassat J-L, Maroteaux L, Borrelli E, Hen R (1990) Cloning and characterization of a *Drosophila* serotonin receptor that activates adenylate cyclase. Proc Natl Acad Sci USA 87: 8940–8944.

Yellman C, Tao H, He B, Hirsh J (1997) Conserved and sexually dimorphic behavioral responses to biogenic amines in decapitated *Drosophila*. Proc Natl Acad Sci USA 94:4131–4136.