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Inhaltsverzeichnis:

Liste der eingebrachten Publikationen:

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[15] SCHLENSTEDT, J., BALFANZ, S., BAUMANN, A. AND **BLENAU, W.** (EINGEREICHT) Am5-HT_7: functional characterization of the first serotonin receptor of the honeybee (*Apis mellifera*). *J. Neurosci.*

Zusammenfassung

Biogene Amine sind kleine organische Verbindungen, die sowohl bei Wirbeltieren als auch bei Wirbellosen als Neurotransmitter, Neuromodulatoren und/oder Neurohormone wirken können. Sie bilden eine bedeutende Gruppe von Botenstoffen und entfalten ihre Wirkungen über die Bindung an eine bestimmte Klasse von Rezeptorproteinen, die als G-Protein-gekoppelte Rezeptoren bezeichnet werden. Bei Insekten gehören zur Substanzklasse der biogenen Amine die Botenstoffe Dopamin, Tyramin, Octopamin, Serotonin und Histamin. Neben vielen anderen Wirkung ist z.B. gezeigt worden, daß einige dieser biogenen Amine bei der Honigbiene (*Apis mellifera*) die Geschmacksempfindlichkeit für Zuckerwasser-Reize modulieren können. Ich habe verschiedene Aspekte der aminergen Signaltransduktion an den „Modellorganismen" Honigbiene und Amerikanische Großschabe (*Periplaneta americana*) untersucht. Aus der Honigbiene, einem „Modellorganismus" für das Studium von Lern- und Gedächtnisvorgängen, wurden zwei Dopamin-Rezeptoren, ein Tyramin-Rezeptor, ein Octopamin-Rezeptor und ein Serotonin-Rezeptor charakterisiert. Die Rezeptoren wurden in kultivierten Säugerzellen exprimiert, um ihre pharmakologischen und funktionellen Eigenschaften (Kopplung an intrazelluläre Botenstoffwege) zu analysieren. Weiterhin wurde mit Hilfe verschiedener Techniken (RT-PCR, Northern-Blotting, *in situ*-Hybridisierung) untersucht, wo und wann während der Entwicklung die entsprechenden Rezeptor-mRNAs im Gehirn der Honigbiene exprimiert werden. Als Modellobjekt zur Untersuchung der zellulären Wirkungen biogener Amine wurden die Speicheldrüsen der Amerikanischen Großschabe genutzt. An isolierten Speicheldrüsen läßt sich sowohl mit Dopamin als auch mit Serotonin Speichelproduktion auslösen, wobei Speichelarten unterschiedlicher Zusammensetzung gebildet werden. Dopamin induziert die Bildung eines völlig proteinfreien, wäßrigen Speichels. Serotonin bewirkt die Sekretion eines proteinhaltigen Speichels. Die Serotonin-induzierte Proteinsekretion wird durch eine Erhöhung der Konzentration des intrazellulären Botenstoffs cAMP vermittelt. Es wurden die pharmakologischen Eigenschaften der Dopamin-Rezeptoren der Schaben-Speicheldrüsen untersucht sowie mit der molekularen Charakterisierung putativer aminerger Rezeptoren der Schabe begonnen. Weiterhin habe ich das *ebony*-Gen der Schabe charakterisiert. Dieses Gen kodiert für ein Enzym, das wahrscheinlich bei der Schabe (wie bei anderen Insekten) an der Inaktivierung biogener Amine beteiligt ist und im Gehirn und in den Speicheldrüsen der Schabe exprimiert wird.

Abstract

Biogenic amines are small organic compounds that act as neurotransmitters, neuromodulators and/or neurohormones in vertebrates and in invertebrates. They form an important group of messenger substances and mediate their diverse effects by binding to membrane receptors that primarily belong to the large gene-family of G protein-coupled receptors. In insects, the group of biogenic amine messengers consists of five members: dopamine, tyramine, octopamine, serotonin, and histamine. Besides many other effects, some of these biogenic amines were shown, for example, to modulate gustatory sensitivity to sucrose stimuli in the honeybee (*Apis mellifera*). I have investigated various aspects of the aminergic signal transduction in the "model organisms" honeybee and American cockroach (*Periplaneta americana*). So far, I have characterized two dopamine receptors, a tyramine receptor, an octopamine receptor and a serotonin receptor of the honeybee, which is well-known for its learning and memory capacities. The receptors where expressed in cultivated mammalian cells in order to analyze their pharmacological and functional (i.e., second messenger coupling) properties. The spatio-temporal expression patterns of the respective receptor mRNA were investigated in the honeybee brain by using different techniques (RT PCR, Northern blotting, *in situ*-hybridization). The salivary glands of the American cockroach were used as a model object in order to investigate the cellular effects of biogenic amines. Both dopamine and serotonin trigger salivary secretion in isolated salivary glands. The quality of the secreted saliva is, however, different. Stimulation of the glands by serotonin results in the production of a protein-rich saliva, whereas stimulation by dopamine results in saliva that is protein-free. Serotonin-induced protein secretion is mediated by an increase in the intracellular concentration of cAMP. The pharmacological properties of dopamine receptors associated with cockroach salivary glands were investigated and the molecular characterization of putative aminergic receptors of the cockroach was initiated. Furthermore, I have characterized the *ebony* gene of the cockroach. This gene encodes an enzyme that is probably involved in the inactivation of biogenic amines in the cockroach (as in other insects). The *ebony* gene is expressed in the brain and in the salivary glands of the cockroach.

1. Einleitung

Zellen müssen extrazelluläre Signale empfangen und darauf reagieren können. Diese Fähigkeiten sind von fundamentaler Bedeutung für das Leben. In mehrzelligen Organismen tauschen Zellen mit unterschiedlichen Funktionen eine Vielzahl von Signalen aus. Signalmoleküle können entweder über beträchtliche Entfernungen transportiert werden und ihre Wirkungen auf entfernte Zielzellen entfalten (z.B. Hormone), oder sie können als lokale Mediatoren wirken und nur Zellen in ihrer unmittelbaren Umgebung beeinflussen (z.B. Neurotransmitter). In allen Fällen wird das Signal durch einen spezifischen Rezeptor wahrgenommen und in eine zelluläre Reaktion umgesetzt. Sowohl die Anzahl biologisch wirksamer Signale als auch die durch sie ausgelösten Reaktionen sind nahezu unüberschaubar. Trotzdem benutzen die Organismen nur relativ wenige, evolutionär hochkonservierte Mechanismen, um extrazelluläre Signale zu empfangen und sie in intrazelluläre Wirkungen umzuwandeln. So können bei der Signaltransduktion über plasmamembranständige Rezeptoren drei grundlegende Mechanismen unterschieden werden:

a) Eine erste Gruppe plasmamembranständiger Rezeptormoleküle bilden die ligandengesteuerten Ionenkanäle oder ionotrope Rezeptoren. Die Bindung eines chemischen Liganden an seinen Rezeptor induziert die Öffnung eines intrinsischen Ionenkanals. Ionotrope Rezeptoren sind an der schnellen synaptischen Signalübertragung zwischen elektrisch erregbaren Zellen beteiligt und können ihre Leitfähigkeit sehr schnell verändern. Die bekanntesten Vertreter sind nikotinische Acetylcholin-Rezeptoren, ionotrope Glutamat-Rezeptoren, Glycin-Rezeptoren, $GABA_A$-Rezeptoren und 5-HT_3-Rezeptoren (ORTELLS & LUNT, 1995).

b) Eine weitere Gruppe sind die G-Protein-gekoppelten Rezeptoren oder metabotropen Rezeptoren (siehe Abb. 1). Diese Rezeptoren besitzen als gemeinsames Strukturmerkmal sieben Transmembran-Domänen (TM). Der N-Terminus der Rezeptoren ist extrazellulär, der C-Terminus intrazellulär lokalisiert. G-Protein-gekoppelte Rezeptoren modulieren indirekt (über GTP-bindende Proteine oder kurz G-Proteine) die Aktivität anderer Zielproteine. Die Aktivierung des Zielproteins führt entweder zu Änderungen in der Konzentration sekundärer Botenstoffe, wenn das Zielprotein ein Enzym ist, oder zu Änderungen in der Permeabilität der Plasmamembran, wenn das Zielprotein ein Ionenkanal ist. Zu den wichtigsten Signalwegen gehören die Erhöhung oder Erniedrigung der intrazellulären cAMP-Konzentration ($[cAMP]_i$) sowie die Mobilisierung von Inositol-1,4,5-trisphosphat mit anschließender Freisetzung von Ca^{2+} aus intrazellulären Speichern. Verglichen mit ionotropen Rezeptoren arbeiten metabotrope Rezeptoren langsamer. Die bekanntesten Vertreter dieser Rezeptorklasse sind das Rhodopsin, muskarinische Acetylcholin-Rezeptoren, α- und β-adrenerge Rezeptoren, und Rezeptoren für Dopamin, Serotonin und eine Reihe von Neuropeptiden (JI ET AL., 1998; MORRIS & MALBON, 1999; **BLENAU & BAUMANN, 2001**).[1]

[1] Die fettgedruckten Literaturverweise kennzeichnen eingebrachte Publikationen.

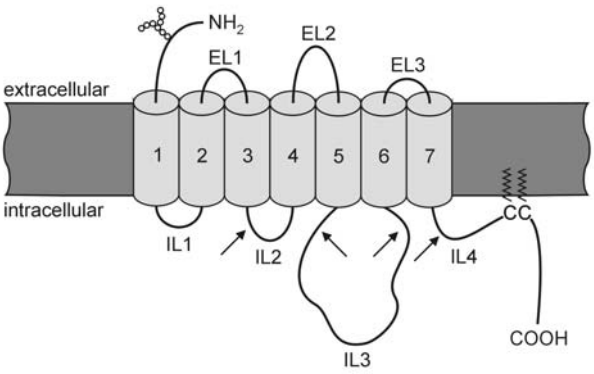

Abbildung 1: Schematische Darstellung eines biogenen Amin-Rezeptors (aus: **BLENAU & BAUMANN, 2003**). Die Polypeptidkette durchspannt die Plasmamembran sieben Mal. Die Transmembran-Domänen (TM 1-7) sind als Zylinder dargestellt. Der N-Terminus (NH_2) befindet sich extrazellulär und enthält häufig glykosylierte Aminosäurereste (o). Der C-Terminus (COOH) befindet sich intrazellulär. Die Transmembran-Domänen sind durch drei extrazelluläre Schleifen (EL) und drei intrazelluläre Schleifen (IL) miteinander verbunden. Wenn das Protein am C-terminalen Enden an Cystein-Resten (C) post-translational palmitoyliert wird, entsteht eine vierte intrazelluläre Schleife (IL4). Die Pfeile weisen auf Rezeptorbereiche, die an der Wechselwirkung mit G-Proteinen beteiligt sind.

c) Eine dritte Gruppe bilden membranständige Rezeptoren, die gleichzeitig Enzyme sind (Rezeptorenzyme oder katalytische Rezeptoren). Es sind meist Proteine, die die Zellmembran einmal durchspannen, extrazellulär eine Ligandenbindungstelle und intrazellulär eine katalytische Domäne besitzen. Diese Rezeptoren sind meist Proteinkinasen, Proteinphosphatasen oder aber Guanylylzyklasen. Beispiele sind der Insulin-Rezeptor (WHITEHEAD ET AL., 2000) sowie Rezeptoren für Wachstumsfaktoren.

Eine bedeutende Gruppe von Botenstoffen, die vorwiegend an G-Protein-gekoppelte Rezeptoren binden, sind die biogenen Amine (VERNIER ET AL., 1995; **BLENAU & BAUMANN, 2001, 2003; BAUMANN ET AL., 2003**). Biogene Amine sind kleine organische Verbindungen, die sowohl bei Wirbeltieren als auch bei Wirbellosen als Neurotransmitter, Neuromodulatoren und/oder Neurohormone wirken können. Ausgangspunkt für die Biosynthese der biogenen Amine sind die essentiellen Aminosäuren Tyrosin, Tryptophan und Histidin. Die Synthese der biogenen Amine erfolgt in ein- bis mehrstufigen Reaktionen. Zur Substanzklasse der biogenen Amine gehören bei Wirbeltieren die Botenstoffe Dopamin, Noradrenalin und Adrenalin, die sich vom Tyrosin ableiten (siehe Abb. 2), Serotonin (5-Hydroxytryptamin, 5HT), das sich vom Tryptophan ableitet, und Histamin, das sich vom Histidin ableitet. Bei Wirbellosen sind die Catecholamine Noradrenalin und Adrenalin durch die Phenolamine Tyramin und Octopamin funktionell ersetzt (siehe Abb. 2; EVANS, 1980; ROEDER, 1999; ROEDER ET AL., 2003; **BLENAU & BAUMANN, 2003**)

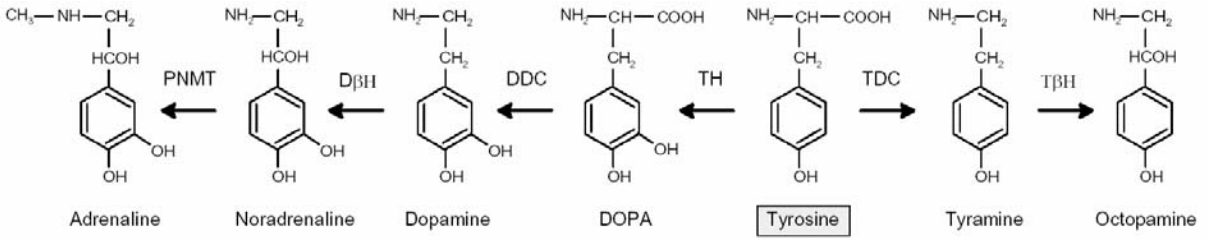

Abbildung 2. Biosynthesewege der biogenen Catecholamine und Phenolamine. Tyrosin ist das Substrat für die Synthese beider Gruppen von Botenstoffen. TH, Tyrosinhydroxylase; DDC, DOPA-Decarboxylase; DβH, Dopamin-β-Hydroxylase; PNMT, Phenylethanolamin-N-Methyltransferase; TDC, Tyrosindecarboxylase; TβH, Tyramin-β-Hydroxylase.

Im Mittelpunkt meines eigenen Interesses steht die molekulare und pharmakologische Charakterisierung von biogenen Amin-Rezeptoren aus der Honigbiene *Apis mellifera* und der Amerikanischen Schabe *Periplaneta americana*. Beide Insektenarten sind wichtige Modellorganismen der neurobiologischen Grundlagenforschung (siehe Abb. 3; BICKER & MENZEL, 1989; DOWNER, 1990; MENZEL & MÜLLER, 1996).

Der Kenntnisstand über die molekularen und funktionellen Eigenschaften von biogenen Amin-Rezeptoren bei Insekten liegt noch immer weit hinter dem Wissen bei Wirbeltieren zurück. Bemerkenswerte Fortschritte wurden in den vergangenen Jahren jedoch nicht nur am Modellinsekt *Drosophila melanogaster* gewonnen. Auch unsere eigenen Arbeiten zur molekularen Klonierung und funktionellen Charakterisierung verschiedener biogener Amin-Rezeptoren der Honigbiene *Apis mellifera* (2 Dopamin-Rezeptoren: BLENAU ET AL., 1998; **MUSTARD ET AL., 2003**; 1 Tyramin-Rezeptor: **BLENAU ET AL., 2000**; **MUSTARD ET AL., 2005** und 1 Octopamin-Rezeptor: **GROHMANN ET AL., 2003**) haben substantiell zum gegenwärtigen Kenntnisstand beigetragen (Übersichten bei: **BLENAU & BAUMANN, 2001, 2003; BAUMANN ET AL., 2003**). Wir haben die pharmakologischen und funktionellen Eigenschaften (Kopplung an sekundäre Botenstoffwege) dieser Rezeptoren sowie die Expressionsmuster der entsprechenden Rezeptor-Gene beschrieben. Mit der Veröffentlichung der kompletten Genom-Sequenz von *Drosophila melanogaster, Anopheles gambiae* und *Apis mellifera* ist abzusehen, dass in naher Zukunft weitere biogene Amin-Rezeptoren dieser Modellorganismen charakterisiert werden (BRODY & CRAVCHIK, 2000; HILL ET AL., 2002). Leider ist bisher nur sehr wenig über die molekularen Eigenschaften von Amin-Rezeptoren bei anderen Insektenarten bekannt.

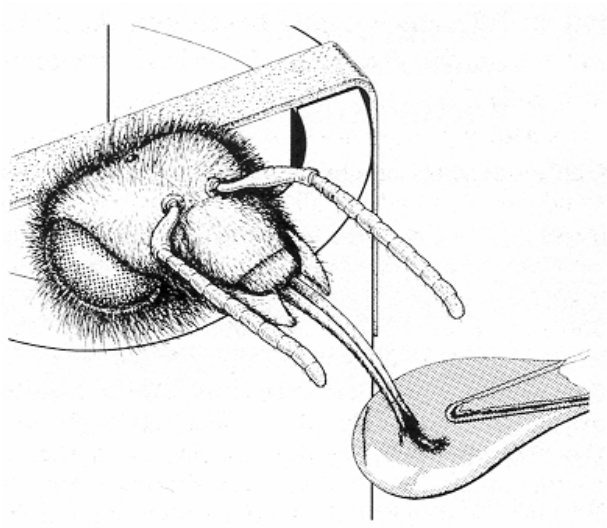

Abbildung 3. Olfaktorische Konditionierung der Rüsselreaktion („proboscis extension response“, PER) bei der Honigbiene, einem wichtigen Modellorganismus für das Studium von Lern- und Gedächtnisvorgängen (aus: MENZEL, 1995). Dabei wird ein Duftreiz („conditioned stimulus“, CS) mit einem Zuckerwasserreiz („unconditioned stimulus“, US) gepaart. Als Folge eines solchen Lernaktes wird der ursprünglich neutrale CS mit dem US assoziiert und löst die Reaktion, das Herausstrecken des Proboscis (PER) aus. Diese Duftassoziation ist sehr effektiv und folgt den Regeln der klassischen Konditionierung.

2. Das dopaminerge System der Honigbiene

Das Catecholamin Dopamin kommt im zentralen Nervensystem (CNS) der Honigbiene in relativ hohen Konzentrationen vor (MERCER ET AL., 1983; FUCHS ET AL., 1989; TAYLOR ET AL., 1992; WAGENER-HULME ET AL., 1999; SCHULZ & ROBINSON, 1999). Dopaminerge Interneuronen verzweigen sehr stark und projizieren in weite Gebiete des Bienengehirns (SCHÄFER & REHDER, 1989; SCHÜRMANN ET AL., 1989; BICKER, 1999; **BLENAU ET AL., 1999**). Das deutet darauf hin, dass Dopamin bei der Biene eine wichtige Rolle als Neurotransmitter und/oder Neuromodulator spielt. Dafür sprechen auch verhaltensphysiologische Experimente: Die Injektion von Dopamin in den α-Lobus der Pilzkörper verändert die elektrische Reaktion auf olfaktorische Reize (MERCER & ERBER, 1983) und reduziert die Rüsselreaktion auf Wasserdampfreize (BLENAU ET AL., 1998). Dopamin beeinflusst auch die Abrufbarkeit des olfaktorischen Gedächtnisses (MERCER & MENZEL, 1982; MACMILLAN & MERCER, 1987). Weil die direkte Injektion von Dopamin (und anderer biogener Amine) unspezifische Reaktionen hervorgerufen haben könnte, haben MENZEL und Mitarbeiter (1999) eine alternative Methode angewendet: Sie haben zuerst Amin-haltige Vesikel durch Behandlung mit Reserpin entleert. Dies führte zu einer starken Beeinträchtigung der Motorik (PER). Die anschließende Injektion von Dopamin in das Gehirn stellte die durch das Reserpin hervorgerufenen Defekte in den motorischen Mustern wieder her. Die Injektion von Dopamin hatte jedoch keine Wirkung auf die Sensitisierung oder Konditionierung der Bienen (MENZEL ET AL., 1999).

2.1 Dopaminerge Innervation der Pilzkörper der Honigbiene und verwandter Hymenopteren

Die Organisation des CNS von Insekten und anderen Arthropoden unterscheidet sich grundlegend von der des CNS von Wirbeltieren. Bei Arthropoden befinden sich die Somata der Nervenzellen in den Ganglien im engeren Sinne, und sind von Regionen zu unterscheiden, in denen die Dendriten und Axone interagieren (Neuropil-Strukturen). Gewöhnlich erhalten die Somata von zentralen Neuronen keine synaptischen Eingänge und sind deshalb wahrscheinlich nicht an der neuronalen Informationsaufnahme beteiligt. Es sind jedoch inzwischen einige Ausnahmen bezüglich dieses Organisationsprinzips beschrieben worden. Dazu zählen dopaminerge Fasern, welche die Somata der intrinsischen Pilzkörperzellen von Arbeiterinnen der Honigbiene innervieren (SCHÄFER & REHDER, 1989; SCHÜRMANN ET AL., 1989; **BLENAU ET AL., 1999).** Die Pilzkörper sind zentral gelegene, paarige und sehr auffällige Strukturen im Gehirn der meisten Arthropoden (siehe Abb. 4). Sie bestehen bei der Biene aus jeweils etwa 170.000 intrinsischen Neuronen **(**Kenyon-Zellen**),** deren Zellkörper im dorsal posterioren Gehirn liegen (WITTHÖFT, 1967; MOBBS, 1982). Ein Pilzkörper gliedert sich in Calyx (Kelch), Pedunculus (Stiel) und mehrere Loben. Die Pilzkörper liegen in einem Nebenzweig der Riechbahn und werden generell mit höheren integrativen Leistungen wie Lernen und Gedächtnis in Verbindung gebracht (ERBER ET AL., 1980; MENZEL ET AL, 1994; HEISENBERG, 2003). Wir haben die Verteilung dopaminerger Nervenfaser in den Pilzkörpern von Arbeiterinnen detailliert analysiert (**BLENAU ET AL., 1999)**. Weiterhin haben wir untersucht, ob dopaminerge Fasern, welche die Zellkörperregion der Kenyon-Zellen innervieren, auf Arbeiterinnen von *Apis mellifera* beschränkt sind, oder ob sie auch bei Drohnen und Königinnen auftreten bzw. auch bei anderen Insekten-Spezies nachgewiesen werden können (**BLENAU ET AL., 1999)**.

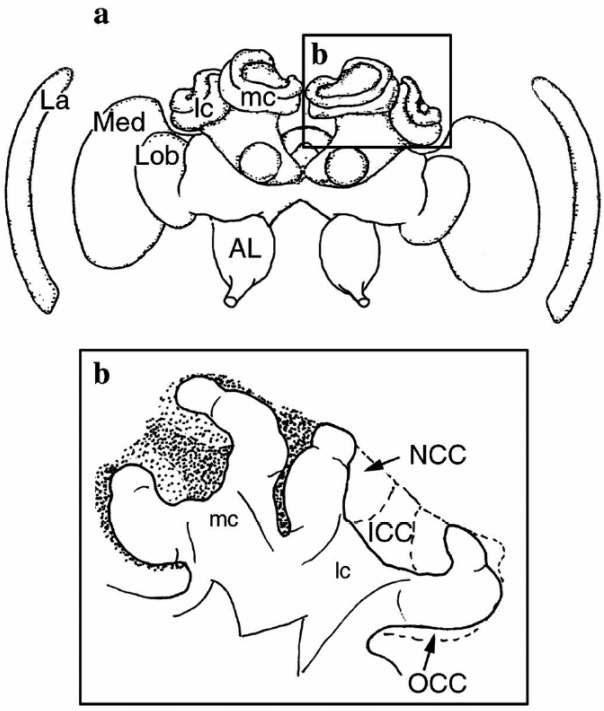

Abbildung 4: a) Schematische Darstellung des Gehirns der Honigbiene (verändert nach: MOBBS, 1985). AL, Antennallobus; La, Lamina; Med, Medulla; Lob, Lobula; lc, lateraler Pilzkörper-Kelch; mc, medianer Pilzkörper-Kelch. **b)** Schematische Darstellung der wichtigsten Kenyon-Zell-Subpopulationen, deren Zellkörper innerhalb und außerhalb des lateralen (lc) und medianen (mc) Kelches des linken Pilzkörpers liegen (verändert nach: KURSHAN ET AL., 2003). OCC, äußere kompakte Zellen; NCC, nicht-kompakte Zellen; ICC, innere kompakte Zellen.

Das dichte Netzwerk dopaminerger Fasern im Pedunculus und in den Loben sowie die schwächere Innervation der Kelche hat seinen Ursprung in einem Faserbündel, das von einer kleinen Gruppe dopaminerger Zellkörper ausgeht. Diese Somata-Gruppe (C_3) liegt unterhalb des lateralen Kelches der Pilzkörper (SCHÄFER & REHDER, 1989; **BLENAU ET AL., 1999**). Innerhalb der Pilzkörperkelche sind die dopaminergen Fasern ungleichmäßig verteilt. Sie besitzen variköse Anschwellungen, was typisch für aminerge Neuronen ist. Dopaminerge Fasern, welche die Zellkörperregion der intrinsischen Pilzkörperzellen innervieren, ziehen aus dem Neuropil der Kelche und Stiele vorwiegend in den Grenzbereich zwischen den nichtkompakten und den inneren kompakten Kenyon-Zellkörpern (siehe Abb. 4; **BLENAU ET AL., 1999**). Innerhalb der Zellkörperregion verlaufen die dopaminergen Fasern ohne auffällige Verzweigungen relativ geradlinig. Durch immunhistochemische Anfärbungen und elektronenmikroskopische Analyse dieser Region konnten immungefärbte Profile nachgewiesen werden, die sich in direktem Kontakt zur Plasmamembran der Kenyon-Zellkörper befinden (**BLENAU ET AL., 1999**). In einigen Fällen konnten Synapsen-ähnliche Strukturen in den Kontaktbereichen zwischen immungefärbten Profilen und den Kenyon-Zellkörpern nachgewiesen werden. Hierbei war in allen Fällen das dopaminerge Profil präsynaptisch zum Kenyon-Zellkörper. Die Polarität dieser synaptischen Verbindung deutet eine modulatorische Wirkung des Dopamins auf die Eigenschaften der intrinsischen Pilzkörperzellen an.

Es bestehen keine auffälligen Unterschiede im Innervationsmuster der Kenyon-Zellkörperregion zwischen Arbeiterinnen, Drohnen und Königinnen (**BLENAU ET AL., 1999**). Dopaminerge Fasern, die den Kenyon-Zellkörperbereich innervieren, konnten nur bei den beiden anderen untersuchten Hymenopteren-Arten, Hornissen (*Vespa crabro*) und Deutschen Wespen (*Vespula germanica*), nicht aber bei Insekten, die zu anderen Ordnungen gehören, nachgewiesen werden (**BLENAU ET AL., 1999**).

2.2 Vergleich der funktionellen und pharmakologischen Eigenschaften von zwei Dopamin-D1-Rezeptoren der Honigbiene – AmDOP1 und AmDOP2

Radioliganden-Bindungsstudien haben gezeigt, dass Dopamin-Rezeptoren im Gehirn der Honigbiene weitverteilt vorkommen, und dass sich die pharmakologischen Eigenschaften dieser Rezeptoren der Biene von denen der Wirbeltiere unterscheiden (BLENAU ET AL., 1995A; KOKAY & MERCER, 1996; KOKAY ET AL., 1998, 1999). Bei Wirbeltieren bindet Dopamin an zwei Unterfamilien von Dopamin-Rezeptoren: D1- und D2-(ähnliche) Rezeptoren (KEBABIAN & CALNE, 1979; SEEMAN & VAN TOL, 1994; MISSALE ET AL., 1998; **BLENAU & BAUMANN, 2001**; CALLIER ET AL., 2003). Diese können anhand ihrer pharmakologischen Eigenschaften und ihrer intrazellulären sekundären Botenstoffe unterschieden werden. Zur Gruppe der D1-Rezeptoren, welche die Adenylyl-Zyklase stimulieren, gehören der D_1- und der D_5-Rezeptor. In der Gruppe der D2-Rezeptoren werden der D_2-, der D_3- und der D_4-Rezeptor zusammengefasst. Für D2-Rezeptoren ist eine Hemmung der Adenylyl-Zyklase oder eine G-Protein-vermittelte Kopplung an andere intrazelluläre Botenstoffwege beschrieben.

Mit der Methode der Homologie-Hybridisierung ist es uns gelungen, aus einer Bienengehirn-cDNA-Bibliothek eine Dopamin-Rezeptor-cDNA (Am*dop*1) zu klonieren sowie funktionell zu charakterisieren (BLENAU ET AL., 1998). Der offene Leserahmen des Am*dop*1-Gens kodiert für ein Protein von 402 Aminosäuren Länge mit einem berechneten Molekulargewicht von 44,6 kDa (AmDOP1; BLENAU ET AL., 1998). Die abgeleitete Aminosäuresequenz des AmDOP1-Rezeptors weist starke Homologie zu D1-Rezeptor-Sequenzen der Wirbeltiere auf (BLENAU ET AL., 1998). Wir haben die cDNA des AmDOP1-Rezeptors in einer menschlichen embryonalen Nieren-Zelllinie (HEK 293) exprimiert und gezeigt, dass die Aktivierung des Rezeptors mit Dopamin oder dem nicht-selektiven Dopamin-Rezeptor-Agonisten 6,7-ADTN zu einem Anstieg der $[cAMP]_i$ führt (BLENAU ET AL., 1998). Aufgrund dieser funktionellen Eigenschaften und des pharmakologischen Profils konnten wir den AmDOP1-Rezeptor in die Unterfamilie der D1-Rezeptoren einordnen (BLENAU ET AL., 1998). Im Gegensatz zu den D1-Rezeptoren der Wirbeltiere, welche die Benzazepine SCH 23390 und SKF 38393 mit hoher Affinität binden, binden diese Liganden an den AmDOP1-Rezeptor mit deutlich geringerer Affinität (BLENAU ET AL., 1998).

Inzwischen wurde eine zweite Dopamin-Rezeptor-cDNA aus der Biene (Am*dop*2) isoliert und charakterisiert (HUMPHRIES ET AL., 2003; **MUSTARD ET AL., 2003**). In den Sequenzdatenbanken konnte bisher keine Wirbeltier-Sequenz gefunden werden, die ortholog zur abgeleiteten AmDOP2-Aminosäuresequenz ist. Die AmDOP2-Sequenz ist am engsten mit α_1-adrenergen Sequenzen der Wirbeltiere verwandt (HUMPHRIES ET AL., 2003). Auch der AmDOP2-Rezeptor aktiviert die Adenylyl-Zyklase, wenn er in *Sf*21-Zellen exprimiert wird (HUMPHRIES ET AL., 2003; **MUSTARD ET AL., 2003**). So ähneln sowohl AmDOP1 als auch

AmDOP2 den D1-Rezeptoren der Wirbeltiere darin, dass ihre Aktivierung zu einem Anstieg der $[cAMP]_i$ führt. Die Aminosäuresequenzen der beiden Honigbienen-Rezeptoren sind jedoch zueinander deutlich verschiedener, als die D1-Rezeptoren (D_1 und D_5) der Wirbeltiere.

Wir haben sowohl AmDOP1 als auch AmDOP2 in *Sf*21-Insektenzellen exprimiert, um die funktionellen und pharmakologischen Eigenschaften dieser beiden Dopamin-Rezeptoren parallel zu untersuchen (**MUSTARD ET AL., 2003**). Northern-Blot-Experimente zeigten, dass die mRNAs beider Rezeptoren in *Sf*21-Zellen ähnlich stark exprimiert wurden (**MUSTARD ET AL., 2003**). Überraschenderweise führte die Expression des AmDOP1-Rezeptors (aber nicht des AmDOP2-Rezeptors) in der Abwesenheit von Agonisten zu einer signifikant erhöhten $[cAMP]_i$ (siehe Abb. 5; **MUSTARD ET AL., 2003**). Der AmDOP1-Rezeptor ist also konstitutiv aktiv. Die konstitutive Aktivität des AmDOP1-Rezeptors konnte durch den Antagonisten *cis*(Z)-Flupenthixol dosisabhängig blockiert werden (siehe Abb. 5; **MUSTARD ET AL., 2003**). Flupenthixol wirkt also als „inverser Agonist". Konstitutive Aktivität ist bereits für D_5-Rezeptoren der Wirbeltiere beschrieben worden (TIBERI & CARON, 1994; SUGAMORI ET AL., 1994; CARDINAUD ET AL., 1997). Unsere Arbeit lieferte jedoch den ersten Hinweis für einen konstitutiv aktiven Dopamin-Rezeptor bei Wirbellosen. Über die physiologische Bedeutung der konstitutiven Aktivität des AmDOP1-Rezeptors kann derzeit nur spekuliert werden. Der Anstieg in der basalen cAMP-Konzentration in Zellen, die konstitutiv aktive Rezeptoren exprimieren, soll zu einer Abnahme des Signal-Rausch-Verhältnisses führen. Daher wurde vorgeschlagen, dass konstitutiv aktive Rezeptoren als An/Aus-Schalter wirken könnten (LEFKOWITZ ET AL., 1993). Es besteht theoretisch auch die Möglichkeit, dass endogene Liganden existieren, die, wie Flupenthixol, als inverse Agonisten wirken. Solche endogenen inversen Agonisten wurden bisher für biogene Amin-Rezeptoren noch nicht identifiziert. Sie wurden aber für andere G-Protein-gekoppelte Rezeptoren beschrieben. Zum Beispiel wirken die endogenen Agouti- und Agouti-verwandte (AgRP) Proteine als inverse Agonisten an Melanocortin-Rezeptoren (SIEGRIST ET AL., 1997; NIJENHUIS ET AL., 2001).

Bei allen Versuchen, in denen wir die Wirkung der Agonisten Dopamin und ADTN auf Zellen, die entweder den AmDOP1-Rezeptor oder den AmDOP2-Rezeptor exprimieren, gemessen haben, zeigte sich, dass beide Agonisten die cAMP-Produktion wirksamer über AmDOP1 als über AmDOP2 stimulieren (**MUSTARD ET AL., 2003)**. Andererseits waren die Antagonisten S(+)-Butaclamol und *cis*(Z)-Flupenthixol bei der Hemmung der cAMP-Reaktion an AmDOP2 wirksamer als an AmDOP1 (**MUSTARD ET AL., 2003)**. Zusammenfassend lässt sich feststellen, dass diese Studie erstmalig Hinweise für einen konstitutiv aktiven Dopamin-Rezeptor bei Wirbellosen lieferte. Weiterhin hat der direkte Vergleich von AmDOP1 mit AmDOP2 gezeigt, dass beide Rezeptoren deutlich unterscheidbare funktionelle und pharmakologische Eigenschaften besitzen.

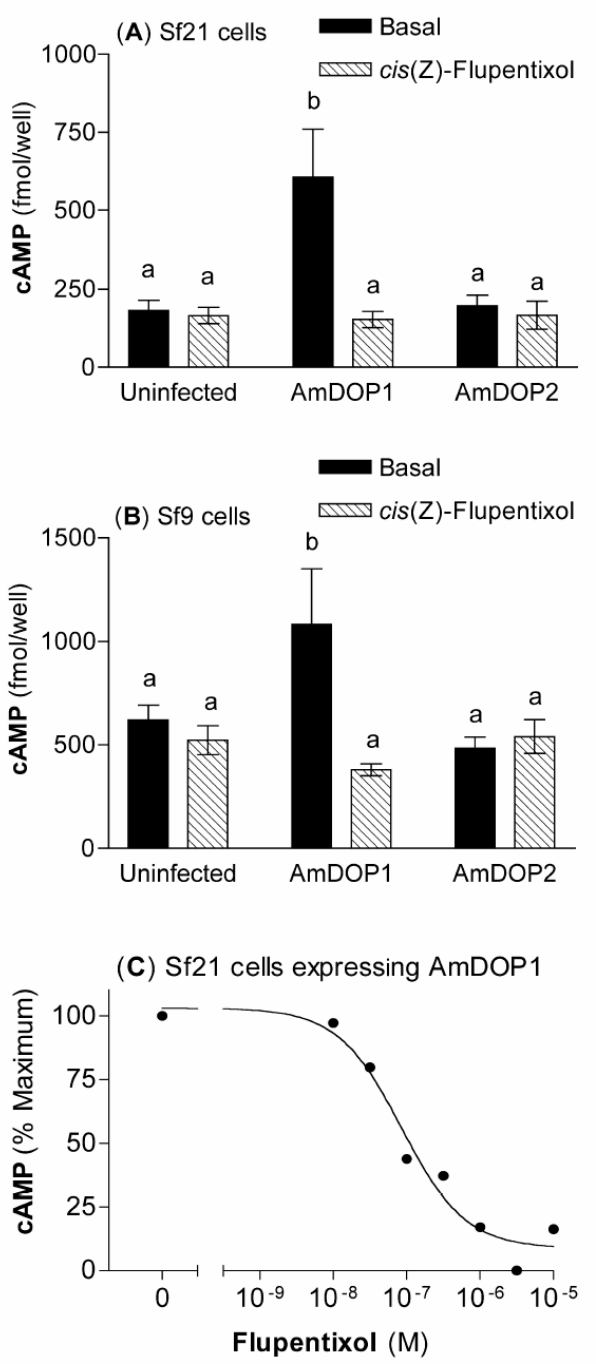

Abbildung 5: Wirkung von Flupenthixol auf die basale intrazelluläre cAMP-Konzentration von nicht-infizierten Zellen (uninfected cells) und Zellen, die AmDOP1- oder AmDOP2-Rezeptoren exprimieren (aus: **MUSTARD ET AL., 2003**). **a)** *Sf*21- oder **b)** *Sf*9-Zellen wurden entweder nur mit IBMX (Basal) oder mit IBMX plus 10^{-5} M *cis*(Z)-Flupenthixol behandelt. Dargestellt sind Mittelwerte ± Standardfehler von sechs unabhängigen Experimenten. **c)** Behandlung von *Sf*21-Zellen, die AmDOP1-Rezeptoren exprimieren, mit IBMX sowie einer Reihe von Flupenthixol-Konzentrationen. Der Punkt „0“ steht für die basale cAMP-Konzentration (in Gegenwart von IBMX). Die gezeigte Kurve ist repräsentativ für drei unabhängige Experimente, die jeweils in Doppelbestimmungen durchgeführt wurden.

3. Der Tyramin-Rezeptor der Honigbiene – AmTYR1

Es wird angenommen, dass bei Wirbellosen die Catecholamine Noradrenalin und Adrenalin durch die strukturell verwandten Phenolamine Tyramin und Octopamin funktionell ersetzt sind. Die Bedeutung von Octopamin als Botenstoff ist etabliert (siehe unten; ROEDER, 1999). Es ist bei Insekten jedoch nur sehr wenig über die physiologische Bedeutung des Tyramins bekannt (KUTSUKAKE ET AL., 2000; NAGAYA ET AL., 2002; BLUMENTHAL, 2002; **BLENAU & BAUMANN, 2003**; ROEDER ET AL., 2003; SARASWATI ET AL., 2004; SCHOLZ, 2005). Bei der Biene können durch die Injektion von Tyramin (oder Octopamin) Reserpin-induzierte Ausfälle in der Rüsselreaktion bei ~50% der behandelten Tiere aufgehoben werden (BRAUN & BICKER, 1992). Darüber hinaus beschleunigt Tyramin in unbehandelten Tieren die Habituationsrate des Reflexes (BRAUN & BICKER, 1992).

3.1 Charakterisierung des AmTYR1-Rezeptors der Honigbiene

Wir haben eine Bienengehirn-cDNA-Bibliothek mit einer cDNA-Sonde des *Drosophila* Tyramin-Rezeptorgens (Dm*tyr*; SAUDOU ET AL., 1990) durchmustert und mehrere cDNA-Klone isoliert. Der längste cDNA-Klon (Am*tyr*1) besteht aus 4.195 Basenpaaren (**BLENAU ET AL., 2000**). Der offene Leserahmen dieses Klons kodiert für ein Protein von 399 Aminosäuren Länge mit einem berechneten Molekulargewicht von 44,7 kDa. Die abgeleitete Aminosäuresequenz ist ca. 80% homolog zu dem als Sonde eingesetzten Tyramin-Rezeptor aus *Drosophila* (DmTYR; SAUDOU ET AL., 1990). Zur Überprüfung des intrazellulären Signalwegs, der von diesem Rezeptor reguliert wird, haben wir das Am*tyr*1-Gen stabil in das Genom von HEK 293-Zellen integriert. Die Aktivierung des Rezeptors durch Tyramin führt zu einer Abnahme der Forskolin-induzierten zelleigenen Adenylyl-Zyklase-Aktivität (siehe Abb. 6; **BLENAU ET AL., 2000**). Der EC_{50}-Wert für Tyramin beträgt ~130 nM. Octopamin ist deutlich weniger effektiv (EC_{50} ~3 µM). Auch wenn der AmTYR1-Rezeptor transient in *Sf*9-Insektenzellen exprimiert wird, führt seine Aktivierung durch Tyramin zur Abnahme der Forskolin-induzierten $[cAMP]_i$ (EC_{50} ~86 nM; **MUSTARD ET AL., 2005**). Hinsichtlich der Kopplung an den intrazellulären Signalweg verhält sich der AmTYR1-Rezeptor wie die Tyramin-Rezeptoren aus *Drosophila* (DmTYR; SAUDOU ET AL., 1990; ROBB ET AL., 1994), *Bombyx mori* (BomOA1, VON NICKISCH-ROSENEGK ET AL., 1996; OHTA ET AL., 2003) und *Locusta migratoria* (LocTYR, VANDEN BROECK ET AL., 1995). Die in HEK 293-Zellen gemessene Abnahme der $[cAMP]_i$ stimmt darüber hinaus sehr gut mit Ergebnissen überein, die an Membranpräparationen aus Bienengehirn gemessen wurden: Die Inkubation mit niedrigen Tyramin-Konzentrationen (0,1-3 µM) inhibiert die cAMP-Synthese (siehe Abb. 7; **BLENAU ET AL., 2000**). Der heterolog exprimierte AmTYR1-Rezeptor reagiert weder auf die Zugabe von Dopamin noch von Serotonin. Das Expressionsmuster des Am*tyr*1-Gens wurde durch *in situ*-Hybridisierungen an Gewebeschnitten des Bienengehirns bestimmt. Starke Expression des Gens wurde in Neuronen der optischen Loben, intrinsischen Pilzkörperneuronen und Neuronen des Deuterocerebrums gefunden (**BLENAU ET AL., 2000**).

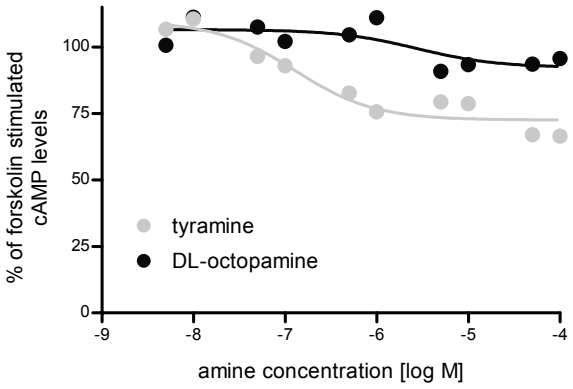

Abbildung 6: Tyramin bewirkt ein Absinken der intrazellulären cAMP-Konzentration in Zellen, die den AmTYR1-Rezeptor exprimieren (aus: **BLENAU ET AL., 2000**). Die cAMP-Konzentration ist als Prozentsatz der Konzentration nach Stimulation mit 20 µM Forskolin (= 100%) dargestellt. Es ist die Wirkung verschiedener Konzentrationen Tyramin und DL-Octopamin auf die Forskolin-induzierte cAMP-Konzentration gezeigt. Dargestellt sind Mittelwerte von mindestens drei unabhängigen Experimenten, die jeweils in Doppelbestimmungen durchgeführt wurden.

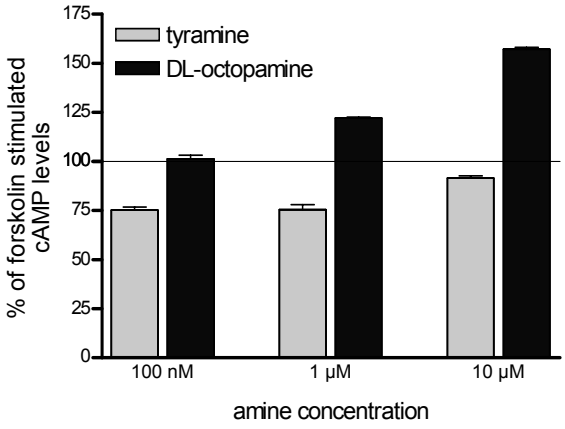

Abbildung 7: Wirkung von Tyramin und DL-Octopamin auf die cAMP-Bildung in Membranhomogenaten aus Bienengehirn (aus: **BLENAU ET AL., 2000**). Es wurde die *de novo*-Synthese von [^{32}P]cAMP nach der Inkubation von Membranhomogenaten aus Bienengehirn mit 10 µM Forskolin (= 100%) und verschiedenen Konzentration Tyramin oder DL-Octopamin gemessen. Dargestellt sind Mittelwerte von zwei unabhängigen Experimenten, die jeweils in Doppelbestimmungen durchgeführt wurden. Fehlerbalken geben Minimal- und Maximalwerte an.

3.2 Entwicklungsabhängige Expression des Am*tyr*1-Gens im Gehirn der Honigbiene

Das Am*tyr*1-Gen wird bereits im sich entwickelnden Gehirn von Honigbienen-Puppen exprimiert (**MUSTARD ET AL., 2005**). Wir haben die Expressionsstärke während verschiedener Entwicklungsstadien durch Northern-Blot-Analyse untersucht (**MUSTARD ET AL., 2005**). Die Expression der 9,5 kb großen Am*tyr*1-mRNA im Gehirn nahm mit dem Alter zu und war in adulten (Pollen-)Sammlerinnen am stärksten (**MUSTARD ET AL., 2005**).

Wir haben das Expressionsmuster des Am*tyr*1-Gens auch in den Somata der sich entwickelnden Kenyon-Zellen der Pilzkörper durch *in situ*-Hybridisierungen detailliert untersucht (**MUSTARD ET AL., 2005**). Subpopulationen von Kenyon-Zellen entstehen während unterschiedlicher, sich überlappender Entwicklungsperioden (FARRIS ET AL., 1999). Sie leiten sich von vier Gruppen von Neuroblasten ab, die bereits in frisch geschlüpften Larven identifiziert werden können (FARRIS ET AL., 1999). Aus diesen zentral gelegenen Proliferationsbereichen gehen konzentrische Schichten von Kenyon-Zellen hervor (siehe auch Abb. 4): Die äußeren kompakten Zellen (OCC) entstehen zuerst und werden durch die nachfolgend entstehenden nicht-kompakten Zellen (NCC) zur Peripherie der Kelche gedrängt. Die NCC werden wiederum durch die inneren kompakten Zellen (ICC) nach außen gedrängt. Die ICC verbleiben im Zentrum eines jeden Kelches. Wir fanden deutliche Änderungen im Expressionsmuster des Am*tyr*1-Gens in den Kenyon-Zellen während unterschiedlicher Entwicklungsstadien (**MUSTARD ET AL., 2005**): Im Puppenstadium (P) 1 (von 9) sind die OCC, die in diesem Stadium unterhalb und seitlich jeder Gruppe von Neuroblasten liegen, intensiv gefärbt. Die NCC, die einen etwas größeren Durchmesser haben, sind nur sehr schwach oder überhaupt nicht gefärbt. Während des P2 sind letztere deutlich gefärbt, wenn auch nicht so intensiv wie die OCC. Ab dem P3-Stadium kehrt sich dieses Verhältnis um. Die Färbung ist jetzt in den sich entwickelnden NCC intensiver als in den OCC. Die ICC, die ab P3 entstehen, sind im P3 nur sehr schwach oder nicht gefärbt. Diese Zellen exprimieren die Am*tyr*1-mRNA erst ab dem P4-Stadium. Dieses Muster bleibt während der verbleibenden Entwicklungsstadien (P5-P9) bis zur Imaginalhäutung stabil. Im Gehirn adulter Bienen ist die Intensität der Färbung in den NCC reduziert. Jetzt erscheint die Färbung in den ICC intensiver als in den OCC und den NCC. Während keines Stadiums der Pilzkörper-Entwicklung ist die Zellproliferationszone gefärbt (**MUSTARD ET AL., 2005**). Insgesamt legen diese Ergebnisse nahe, dass der AmTYR1-Rezeptor eine Funktion bei der Reifung der Pilzkörper haben könnte (**MUSTARD ET AL., 2005**).

4. Der Octopamin-Rezeptor der Honigbiene – AmOA1

Das Phenolamin Octopamin wurde im Gehirn der Honigbiene mit verschiedenen Methoden nachgewiesen (MERCER ET AL., 1983; HARRIS & WOODRING, 1992; KREISSL ET AL., 1994). Die Octopamin-Konzentration im Gehirn von Sammlerinnen ist größer als die im Gehirn von Ammenbienen (WAGENER-HULME ET AL., 1999). Dieser Unterschied ist in den Antennalloben, den ersten zentralen Integrationszentren für Duftreize (siehe Abb. 4), besonders deutlich (SCHULZ & ROBINSON, 1999; SCHULZ ET AL., 2003). Octopamin steuert oder moduliert bei der Biene verschiedene Verhaltensmuster (Übersichten bei: BICKER & MENZEL, 1989; ERBER ET AL., 1993; **BLENAU & BAUMANN, 2001**, **2003**; SCHULZ ET AL., 2002A), wie z.B. die Rüsselreaktion (MERCER & MENZEL, 1982; BRAUN & BICKER, 1992), die

Stachelreaktion (BURRELL & SMITH, 1995), das Erkennen von „Stockgenossen“ (ROBINSON ET AL., 1999), das Antwortverhalten auf Zuckerwasserreize (**SCHEINER ET AL., 2002**) und andere für das Sammelverhalten relevante Reize (BARRON ET AL., 2002) sowie die Arbeitsteilung (SCHULZ & ROBINSON, 2001; SCHULZ ET AL., 2002B, 2003; BARRON & ROBINSON, 2005). Die Injektion von Octopamin in verschiedene Bereiche des CNS verstärkt die neuronale Aktivität und erleichtert die Auslösung von Reflexen (ERBER ET AL., 1993; ERBER & KLOPPENBURG, 1995; KLOPPENBURG & ERBER, 1995; PRIBBENOW & ERBER, 1996). Ein identifiziertes octopaminerges Neuron, VUM_{mx1}, spielt eine wichtige Rolle bei der Repräsentation des Zuckerwasserreizes (US) während der olfaktorischen Konditionierung (siehe Abb. 3, HAMMER, 1993). Das VUM_{mx1}-Neuron reagiert mit Depolarisation auf die Präsentation eines Zuckerwasserreizes an Antenne und Proboscis. Wird das VUM_{mx1}-Neuron mit einer intrazellulären Elektrode kurz nach Beginn einer Duftstimulation depolarisiert, so kann dies den Zuckerwasserreiz während der olfaktorischen Konditionierung ersetzen (HAMMER, 1993). Die gleiche Wirkung wird durch die Injektion von Octopamin in die Antennalloben oder die Kelche der Pilzkörper erzielt (HAMMER & MENZEL, 1998). Es wurde postuliert, dass Octopamin selektiv die „Verstärkerfunktion“, aber nicht die sensitisierende oder reflexauslösende Funktion des Zuckerwasserreizes vermittelt. (MENZEL ET AL., 1999). Die Injektion des Octopamin-Rezeptor-Antagonisten Mianserin in den Antennallobus hemmt die Akquisition und den Abruf des olfaktorischen Gedächtnisses (FAROOQUI ET AL., 2003).

In Membranhomogenaten aus Bienengehirn stimuliert Octopamin die cAMP-Produktion (**BLENAU ET AL., 2000**). Die Injektion von Octopamin in den Antennallobus der Honigbiene bewirkt eine schnelle und transiente Aktivierung der Proteinkinase A (PKA) (HILDEBRANDT & MÜLLER, 1995). Die pharmakologischen Eigenschaften nativer neuronaler Octopamin-Rezeptoren der Biene wurden detailliert untersucht (DEGEN ET AL., 2000) und die Verteilung von [^{3}H]Octopamin-Bindungsstellen im Bienengehirn wurde mit autoradiografischen Methoden analysiert (ERBER ET AL., 1993). Eine hohe Dichte spezifischer Bindungsstellen konnte in den Pilzkörpern, besonders im Pedunculus und den Loben, nachgewiesen werden. Interessanterweise werden diese Gehirnbereiche nicht durch octopaminerge Nervenfasern innerviert (ERBER ET AL., 1993; KREISSL ET AL., 1994). Die spezifische [^{3}H]Octopamin-Bindung wird in allen Gehirnbereichen (außer den Pilzkörpern) zu ~93% durch Phentolamin verdrängt. In den Pilzkörpern kann Phentolamin nur ~70% der spezifischen Bindung verdrängen. Daher wird angenommen, dass sich die Octopamin-Rezeptoren in den Pilzkörpern pharmakologisch von den Rezeptoren in anderen Gehirnbereichen unterscheiden (ERBER ET AL., 1993).

Wir haben einen cDNA-Klon isoliert, der für einen funktionell aktiven Octopamin-Rezeptor kodiert (**GROHMANN ET AL., 2003**). Die cDNA (Am*oa*1) besteht aus 3.023 Basenpaaren. Der längste offene Leserahmen kodiert für ein Protein von 587 Aminosäuren Länge. Es besitzt ein berechnetes Molekulargewicht von 66,5 kDa. Die abgeleitete Aminosäuresequenz besitzt große Ähnlichkeit zu Octopamin-Rezeptorsequenzen aus *Drosophila melanogaster* (HAN ET AL., 1998; BALFANZ ET AL., 2005) und *Lymnea stagnalis* (GERHARDT ET AL., 1997). Wir haben das Am*oa*1-Rezeptorgen heterolog in HEK 293-Zellen exprimiert, um die Kopplung des Rezeptors an intrazelluläre Signalwege aufzuklären (**GROHMANN ET AL., 2003**). Die Zugabe nanomolarer Konzentrationen (≥1 nM) Octopamin führt zur Erhöhung der intrazellulären Ca^{2+}-Konzentration ($[Ca^{2+}]_i$). Die Änderung der $[Ca^{2+}]_i$ haben wir an einzelnen Zellen mit der Ca^{2+}-„imaging“-Technik verfolgt (siehe Abb. 8;

GROHMANN ET AL., 2003). Eine besonders interessante Beobachtung war, dass die Aktivierung der AmOA1-Rezeptoren bei niedrigen Octopamin-Konzentrationen oszillierende Ca^{2+}-Signale in den Zellen auslöste. Ähnliche Reaktionen sind auch für metabotrope Glutamat-Rezeptoren beschrieben worden (KAWABATA ET AL., 1996). Es wurde berichtet, dass durch die oszillierende Ca^{2+}-Konzentrationsänderung die Aktivität bestimmter Gene moduliert werden kann (DOLMETSCH ET AL., 1998). Wenn die Inkubation der Am*oa*1-transfizierten Zellen mit mikromolaren Octopamin-Konzentrationen durchgeführt wurde, kam es zu einer einmaligen, wahrscheinlich vollständigen und irreversiblen Entleerung der intrazellulären Ca^{2+}-Speicher.

Neben der Freisetzung von Ca^{2+} aus intrazellulären Speichern bewirkt die Zugabe von ≥1 µM Octopamin zusätzlich eine geringfügige Erhöhung der $[cAMP]_i$ (**GROHMANN ET AL., 2003**). Wir denken, dass dieser Effekt durch Ca^{2+}/CaM-abhängige Adenylyl-Zyklasen erfolgt, die durch die massive Ca^{2+}-Freisetzung in den transfizierten Zellen als Sekundärantwort aktiviert werden. Die Erhöhung der $[Ca^{2+}]_i$ und der $[cAMP]_i$ ist auch für das orthologe *Drosophila*-Octopamin-Rezeptorgen (OAMB) beschrieben worden (HAN ET AL., 1998). Im Gegensatz zu Octopamin führte die Aktivierung des AmOA1-Rezeptors durch Tyramin nur bei hohen Ligandenkonzentrationen zu Änderungen der intrazellulären Ca^{2+}-Konzentrationen.

Das Expressionsmuster des Am*oa*1-Gens wurde durch *in situ*-Hybridisierungen an Gewebeschnitten von Gehirnen adulter Sammlerinnen untersucht (**GROHMANN ET AL., 2003**). Spezifische Markierungen wurden in vielen Bereichen des CNS (z.B. intrinsische Pilzkörperzellen, Zellen der Antennalloben und der optischen Loben) gefunden. Besonders ausgeprägt war die Markierung in den Somata intrinsischer Pilzkörperzellen. Die Expression des Am*oa*1-Gens in den einzelnen Somata dieser Zellkörperregion ist jedoch unterschiedlich stark. Eine ähnliche Beobachtung wurde auch für den orthologen *Drosophila*-Octopamin-Rezeptor OAMB beschrieben (HAN ET AL., 1998).

Inzwischen haben FAROOQUI und Mitarbeiter (2003) auf der Suche nach möglichen Funktionen des AmOA1-Rezeptors in den Antennalloben erfolgreich die RNAi-Technik angewendet. Die Injektion von AmOA1-dsRNA in den Antennallobus reduziert die Expression des AmOA1-Proteins. Wenn die Injektion der dsRNA 24 h vor einer olfaktorischen Konditionierung erfolgte, führte dies zu einer ca. 80%igen Hemmung in der Akquisition und zu einer ca. 50%igen Hemmung im Abruf des olfaktorischen Gedächtnisses (FAROOQUI ET AL, 2003).

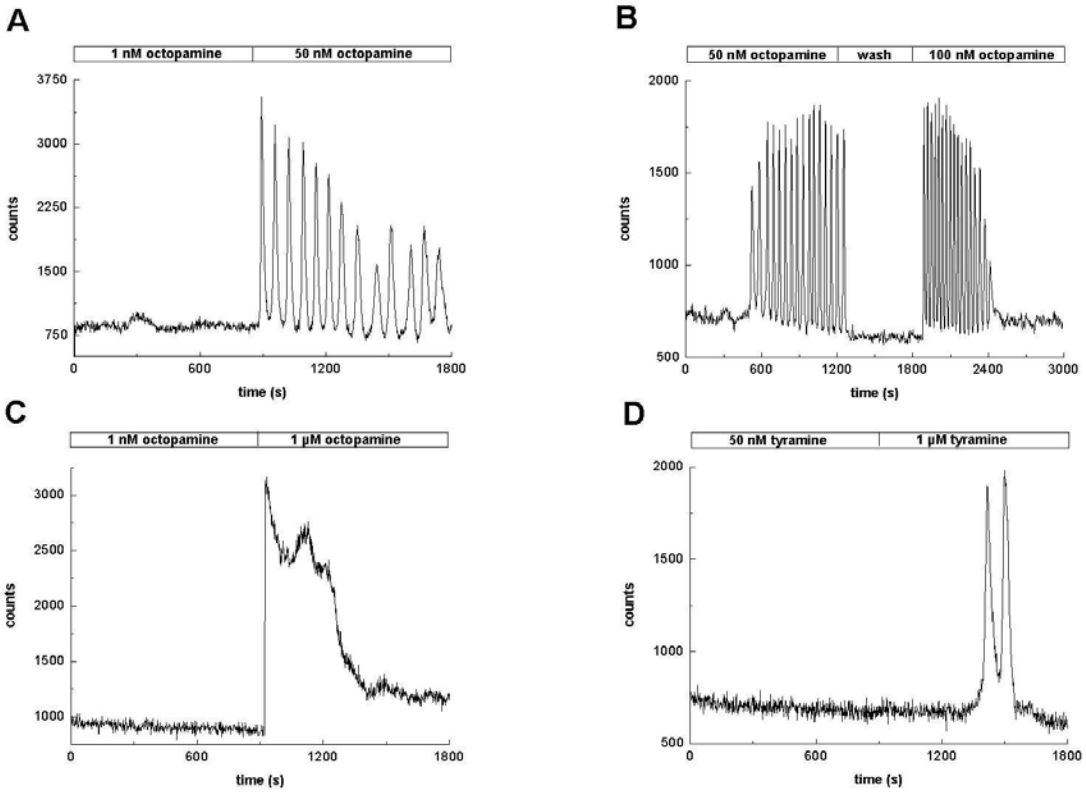

Abbildung 8: Modulation der $[Ca^{2+}]_i$ in Am*oa*1-HA-transfizierten HEK 293-Zellen durch Agonisten (aus: **GROHMANN ET AL., 2003**). Octopamin induziert transiente Erhöhungen in der $[Ca^{2+}]_i$. Gezeigt sind die Ca^{2+}-Reaktionen einzelner, AmOA1-exprimierender Zellen während der Stimulation mit Octopamin oder Tyramin. Die Zellen wurden mit dem Ca^{2+}-empfindlichen Farbstoff Fluo-4 beladen. Die relative Fluoreszenz-Intensität ist als „counts" gegen die Zeit [ms] aufgetragen. Die Zugabe des Liganden ist über dem Signal angegeben. In den folgenden Beschreibungen ist die Anzahl reagierender Zellen und in Klammern die Gesamtanzahl getesteter Zellen angegeben. (A) Zugabe von 1 nM und 50 nM Octopamin (10/13). (B) Zugabe von 50 nM Octopamin. Anschließend wurde mit einer Liganden-freien Lösung gewaschen und danach 100 nM Octopamin zugegeben (5/5). (C) Zugabe von 1 nM und 1 µM Octopamin. Bei einer Konzentration von ≥50 nM treten Oszillationen der $[Ca^{2+}]_i$ auf. Dagegen verursacht 1 µM Octopamin einen starken Anstieg in der $[Ca^{2+}]_i$, der nur langsam zurückgeht (4/4). (D) Zugabe von 50 nM und 1 µM Tyramin (5/7).

5. Der Serotonin-5-HT$_7$-Rezeptor der Honigbiene

Die Verteilung von Serotonin-immunreaktiven Neuronen ist im Gehirn adulter Arbeiterinnen (SCHÜRMANN & KLEMM, 1984; NÄSSEL, 1985; SCHÄFER & BICKER, 1986; REHDER ET AL., 1987) und Honigbienen-Puppen (SEIDEL & BICKER, 1996) untersucht worden. Von insgesamt nur ~75 serotonergen Zellkörpern gehen neuronale Verzweigungen aus, welche jedoch die meisten Neuropil-Gebiete des Gehirns innervieren. Die serotonergen Neuronen im adulten Tier bestehen sowohl aus persistierenden embryonalen Neuronen als auch aus Neuronen, die sich während der Metamorphose differenzieren. Verhaltensexperimente haben gezeigt, dass Serotonin häufig rhythmisches Verhalten und Reflexe reduziert oder blockiert und damit funktionell antagonistisch zum Octopamin wirkt (ERBER ET AL., 1993; ERBER & KLOPPENBURG, 1995; KLOPPENBURG & ERBER, 1995; PRIBBENOW & ERBER, 1996; BLENAU & ERBER, 1998). Serotonin zeigt auch deutliche Wirkung auf das Lernen und die Gedächtnisbildung, indem es sowohl die Akquisition als auch den Abruf erlernten Verhaltens beeinträchtigt (Übersichten bei: BICKER & MENZEL, 1989; MENZEL ET AL., 1994). Serotonin-empfindliche [^{3}H]LSD-Bindungsstellen sind in Membranpräparationen des Bienengehirns charakterisiert worden (BLENAU ET AL., 1995B). Die pharmakologischen Eigenschaften dieser potentiellen Serotonin-Rezeptoren unterscheiden sich deutlich von den Eigenschaften der Wirbeltier-Serotonin-Rezeptoren (BLENAU ET AL., 1995B). Dies macht eine Klassifikation nach dem Schema der Wirbeltier-Serotonin-Rezeptoren auf der Grundlage pharmakologischer Eigenschaften unmöglich.

Wir haben degenerierte Primer, die von konservierten Sequenzabschnitten innerhalb der TM6- und TM7-Segmente abgeleitet sind, für PCR-Experimente mit Bienengehirn-cDNA eingesetzt. Dabei wurde ein Fragment amplifiziert, dessen Sequenz große Ähnlichkeit mit Serotonin-Rezeptorgenen (5-HT$_7$) von *Drosophila melanogaster* (WITZ ET AL., 1990) und *Aedes aegypti* (PIETRANTONIO ET AL., 2001) besitzt. Wir haben mit diesem Fragment eine Bienengehirn-cDNA-Bibliothek durchmustert und einen vollständigen cDNA-Klon (Am*5*-*ht*7) isoliert (**SCHLENSTEDT ET AL., EINGEREICHT**). Abbildung 8 zeigt einen Vergleich der abgeleiteten Aminosäuresequenzen des Am5-HT$_7$-Rezeptors mit 5-HT$_7$-Rezeptor-Sequenzen von *Drosophila* (~52% Ähnlichkeit) und *Aedes*. Mit RT-PCR-Experimenten konnten wir zeigen, dass Am*5*-*ht*7-mRNA im Zentralgehirn, in den optischen Loben, in der Flugmuskulatur und in den Malpighi-Gefäßen der Biene exprimiert wird (**SCHLENSTEDT ET AL., EINGEREICHT**). Durch *in situ*-Hybridisierung wurde Rezeptor-mRNA in den Somata vieler Gehirnregionen nachgewiesen (**SCHLENSTEDT ET AL., EINGEREICHT**). Ein affinitätsgereinigtes Antiserum, das gegen den C-Terminus des Rezeptors gerichtet ist, markiert auf Western-Blots mit Bienengehirn-Proteinen spezifisch eine Bande der erwarteten Größe von ~66 kDa (**SCHLENSTEDT ET AL., EINGEREICHT**).

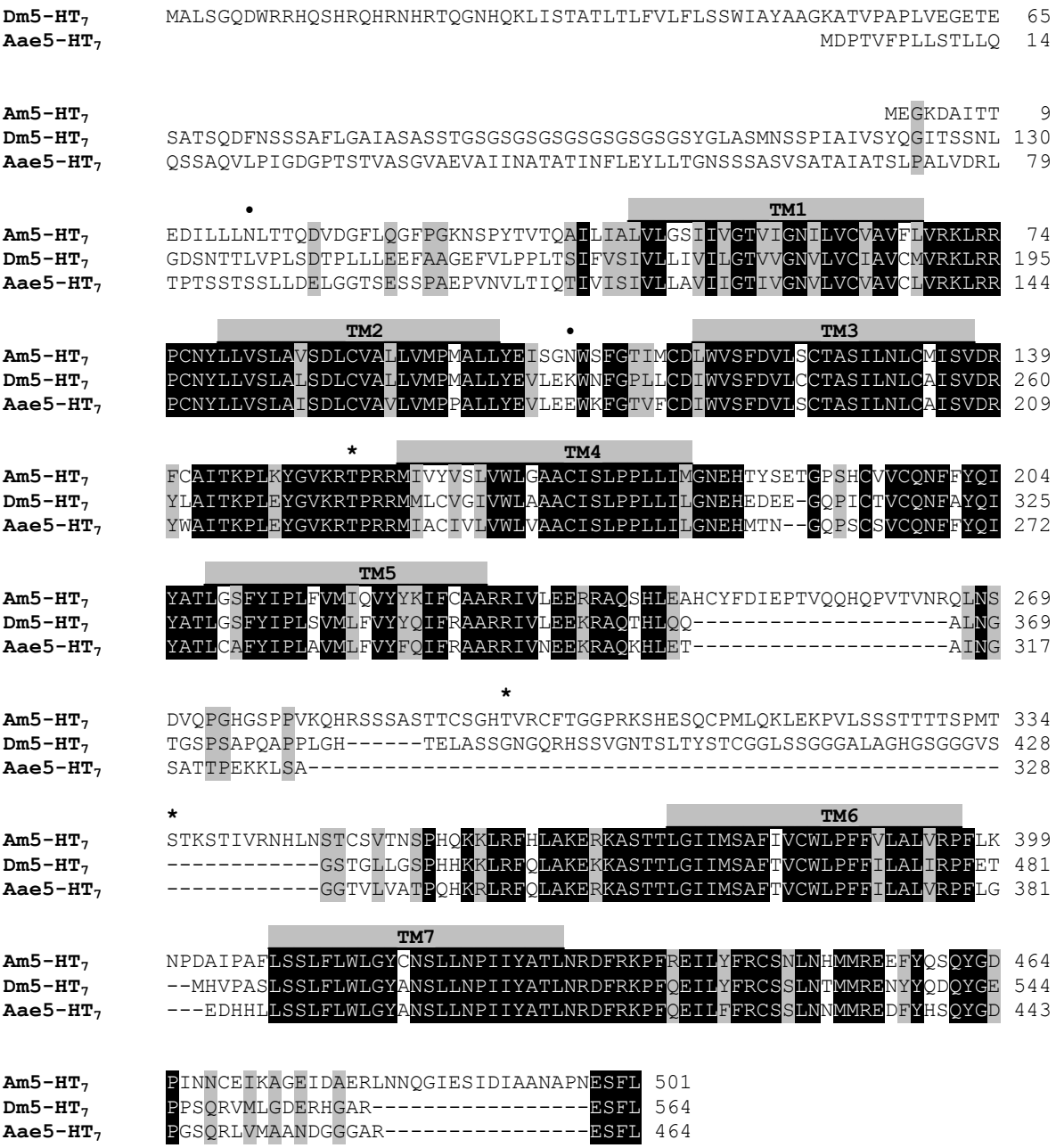

Abbildung 9: Vergleich der abgeleiteten Aminosäuresequenz von Am5-HT$_7$ mit den Sequenzen der 5-HT$_7$-Rezeptoren aus *Drosophila melanogaster* (WITZ ET AL., 1990) und *Aedes aegypti* (PIETRANTONIO ET AL., 2001) (aus: **SCHLENSTEDT ET AL., EINGEREICHT**). Die Transmembran-Segmente sind mit TM1-TM7 markiert. Die Position des letzten Restes jeder Zeile ist am rechen Rand angegeben. Lücken, die zur Maximierung der Homologien eingefügt wurden, sind durch Bindestriche gekennzeichnet. In allen drei Sequenzen identische Aminosäuren sind schwarz unterlegt, konservierte Aminosäureaustausche sind grau unterlegt.

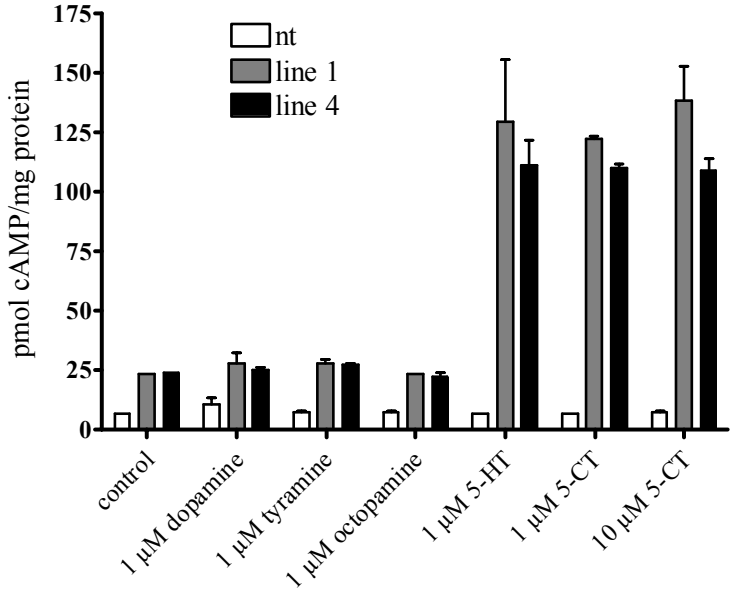

Abbildung 10: Serotonin und der Serotonin-Rezeptor-Agonist 5-CT bewirken einen Anstieg in der intrazellulären cAMP-Konzentration in HEK 293-Zellen, die den Am5-HT_7-Rezeptor stabil exprimieren („line 1" und „line 4"; aus: **SCHLENSTEDT ET AL., EINGEREICHT**). Als Kontrolle wurden nicht-transfizierte HEK 293-Zellen eingesetzt (nt). Die cAMP-Konzentration ist in pmol cAMP/mg Protein dargestellt. Es sind die Wirkungen verschiedener biogener Amine sowie des Agonisten 5-CT auf die cAMP-Produktion gezeigt. Dargestellt sind Mittelwerte von zwei Experimenten, die in Doppelbestimmungen durchgeführt wurden.

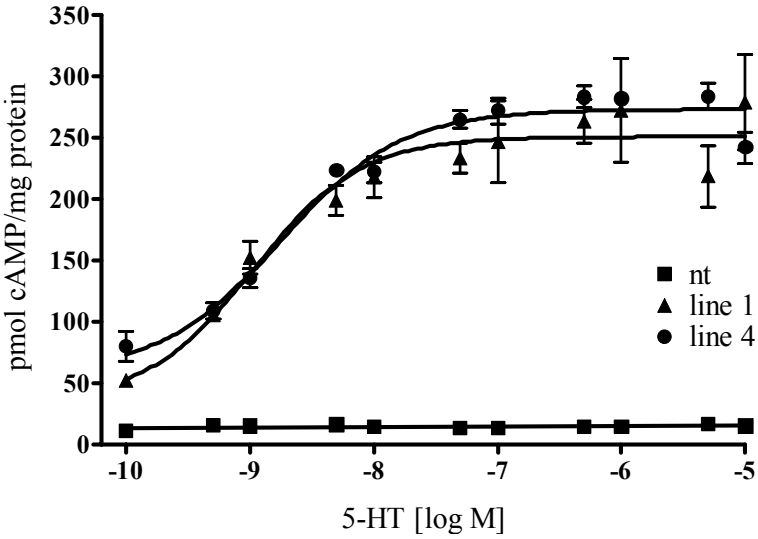

Abbildung 11: Stimulierung der cAMP-Bildung in HEK 293-Zellen, die den Am5-HT_7-Rezeptor stabil exprimieren („line 1" und „line 4"), durch verschiedene Konzentrationen Serotonin (aus: **SCHLENSTEDT ET AL., EINGEREICHT**). Als Kontrolle wurden nicht-transfizierte HEK 293-Zellen eingesetzt (nt). Die cAMP-Konzentration ist in pmol cAMP/mg Protein dargestellt. Es sind Mittelwerte von zwei Experimenten, die in Doppelbestimmungen durchgeführt wurden, angegeben.

Wir haben das Am*5-ht*7-Gen mit einem Haemagglutinin A (HA)-Epitop modifiziert und stabil in das Genom von HEK 293-Zellen integriert (Linie 1 und Linie 2). Auf Western-Blots von Proteinenextrakten dieser transfizierten Zellen ist das Am5-HT_7-HA-Protein nachweisbar (**SCHLENSTEDT ET AL., EINGEREICHT**). Serotonin und der Serotonin-Rezeptor-Agonist 5-CT bewirken einen Anstieg in der intrazellulären cAMP-Konzentration in beiden Am5-HT_7-exprimierenden Zelllinien (siehe Abb. 10; **SCHLENSTEDT ET AL., EINGEREICHT**). Die EC_{50}-Werte für Serotonin betragen 1,1 nM (Linie 1) bzw. 1,8 nM (Linie 4) (siehe Abb. 11; **SCHLENSTEDT ET AL., EINGEREICHT**). Die Serotonin-vermittelte cAMP-Produktion konnte durch Methiotepin, einem Antagonisten an 5-HT_1-, 5-HT_6- und 5-HT_7-Rezeptoren der Säuger, unterdrückt werden (**SCHLENSTEDT ET AL., EINGEREICHT**).

6. Einfluss der biogenen Amine Dopamin, Tyramin und Octopamin auf gustatorische Reaktionsschwellen der Honigbiene

Bei der Honigbiene korreliert die sensorische Empfindlichkeit für Zuckerwasserreize mit vielen Verhaltensparametern wie z.B. dem Alter beim ersten Sammelflug, der Sammelaufgabe (Nektar, Pollen oder beides) und dem Lernen (Übersicht bei: PAGE & ERBER, 2002). Diese Korrelationen machen die Zuckerwasser-Empfindlichkeit zu einem guten Indikator für den Verhaltenszustand einer Biene. Die Zuckerwasser-Empfindlichkeit kann leicht bestimmt werden, indem man der Biene Lösungen ansteigender Saccharose-Konzentration bietet und die Rüsselreaktion (siehe Abb. 3) misst. Die geringste Saccharose-Konzentration, die eine Rüsselreaktion auslöst, ist ein Maß für die individuelle Zuckerwasser-Reaktionsschwelle (PAGE ET AL., 1998). Bisher sind die Mechanismen, die der Regulation und Modulation der Zuckerwasser-Empfindlichkeit zu Grunde liegen, weitgehend unbekannt. Wir haben untersucht, ob die Zuckerwasser-Empfindlichkeit durch die biogenen Amine Octopamin, Tyramin und Dopamin sowie den Dopamin-Rezeptor-Agonisten 6,7-ADTN moduliert werden kann (**SCHEINER ET AL., 2002**). Die Substanzen wurden entweder in den Thorax injiziert oder mit einer Rohrzucker-Lösung verfüttert. Diese Vorgehensweise ermöglichte den Vergleich verschiedener Applikationsmethoden.

Sowohl die Injektion als auch die Verfütterung von Octopamin oder Tyramin erhöht signifikant die Zuckerwasser-Empfindlichkeit (**SCHEINER ET AL., 2002**). Ein Anstieg in der Empfindlichkeit des gustatorischen Systems durch exogen appliziertes Octopamin und Tyramin wurde auch von anderen Autoren gezeigt (LONG & MURDOCK, 1983; BICKER & MENZEL, 1989; BRAUN & BICKER, 1992; PANKIW & PAGE, 2003). Es ist bisher nicht geklärt, ob exogen appliziertes Tyramin *in vivo* zu Octopamin umgesetzt wird oder direkt über die Bindung an spezifische Tyramin- (oder Octopamin-)Rezeptoren wirkt. Dopamin senkt die gustatorische Empfindlichkeit, wenn es in den Thorax injiziert wird (**SCHEINER ET AL., 2002**). Das Verfüttern von Dopamin bewirkt keine Verhaltensänderung. Das synthetische Dopamin-Analogon 6,7-ADTN, das mit hoher Affinität sowohl an den AmDOP1- als auch an den AmDOP2-Rezeptor bindet (BLENAU ET AL., 1998; **MUSTARD ET AL., 2003**), senkt die Zuckerwasser-Empfindlichkeit signifikant (**SCHEINER ET AL., 2002**). Diese Wirkung ist unabhängig davon, ob ADTN injiziert oder verfüttert wird. Diese Ergebnisse zeigen, dass die Zuckerwasser-Empfindlichkeit von Honigbienen durch biogene Amine moduliert werden kann, und die Amine damit wahrscheinlich auch andere Verhaltensweisen der Biene beeinflussen.

7. Die Speicheldrüsen der Schabe *Periplaneta americana* als Modellobjekt zur Untersuchung der zellulären Wirkungen biogener Amine

7.1 Einführung in die Thematik

Ein seit langem etabliertes Modellobjekt zur Untersuchung der zellulären Wirkungen biogener Amine sind die Speicheldrüsen von Schaben und anderen Insekten (HOUSE & GINSBORG, 1985; ALI, 1997; ZIMMERMANN & WALZ, 2003; **WALZ ET AL., 2006**). Amerikanische Schaben (*Periplaneta americana*) besitzen innervierte, acinöse Speicheldrüsen, die aus den sekretorischen Acini und einem Gangsystem bestehen (siehe Abb. 12; JUST & WALZ, 1994). Die Drüsenacini enthalten zwei Zelltypen, periphere (P-) und zentrale (C-)Zellen, die verschiedene Komponenten des Speichels sezernieren und selektiv durch die biogenen Amine Dopamin und/oder Serotonin stimuliert werden (siehe Abb. 12). Experimente an isolierten Speicheldrüsen haben gezeigt, dass Dopamin die Bildung eines völlig proteinfreien, wässrigen Speichels induziert (JUST & WALZ, 1996; **MARG ET AL., 2004**). Serotonin induziert die Sekretion eines proteinhaltigen Speichels (JUST & WALZ, 1996). Den Drüsenacini schließt sich ein verzweigtes Gangsystem (Ausführgänge) an, in dem der Primärspeichel modifiziert wird. Die Gangzellen sind ebenfalls durch Dopamin stimulierbar (LANG & WALZ, 1999, 2001) und modifizieren den Primärspeichel (RIETDORF ET AL., 2003). Sowohl in den Elektrolyt- und Wasser-sezernierenden P-Zellen der Drüsenacini als auch in den Ausführgangzellen bewirkt Dopamin einen Anstieg der $[Ca^{2+}]_i$, der auf bisher ungeklärte Weise den transepithelialen Na^+-, K^+- und Cl^--Transport bewirkt (**WALZ ET AL., 2006**).

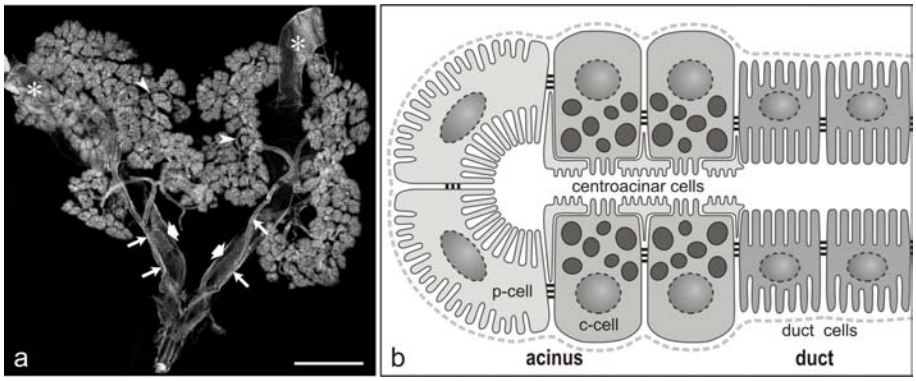

Abbildung 12: Morphologie der Speicheldrüse von *Periplaneta americana* (verändert nach: BAUMANN ET AL., 2002). **a)** Dunkelfeldaufnahme des Speicheldrüsenkomplexes. Die paarigen Speicheldrüsen bestehen aus mehreren Loben acinösen Gewebes. Die Ausführgänge (Pfeilspitzen), die aus den Acini hervorgehen, fusionieren zu einem einzigen Speichelgang (Pfeile) pro Drüse. Die paarigen Reservoire (Sterne) öffnen sich in die Reservoirgänge (breite Pfeile). Balken, 2 mm. **b)** Schematische Darstellung eines Acinus. Jeder Acinus besteht aus zwei peripheren Zellen (p-cells) mit langen Mikrovilli, ungefähr acht zentralen Zellen (c-cells), die sekretorische Granula enthalten, und Zentroacinarzellen (centroacinar cells), die die luminale Oberfläche der C-Zellen auskleiden. Die Ausführgangzellen (duct cells) besitzen sowohl auf ihrer basalen als auch ihrer apikalen Seite tiefe Einfaltungen. *Septate junctions* (dreifache schwarze Linien) verbinden die benachbarten Zellen.

Die Speicheldrüsen von Schaben werden sowohl vom Unterschlundganglion als auch vom stomatogastrischen Nervensystem innerviert (ELIA ET AL., 1994; ALI., 1997; **WALZ ET AL., 2006**). Zwei paarige Neuronen (SN1 und SN2), deren Somata im Unterschlundganglion liegen, senden ihre Axone contralateral (SN1) bzw. ipsilateral (SN2) zum paarigen Speichelgangnerven (Nerv 7b). Die Speichelgangnerven projizieren zum Speicheldrüsenkomplex und verzweigen über den sekretorischen Acini (ALI ET AL., 1993). Das SN1-Neuron ist dopaminerg und stellt wahrscheinlich die einzige Quelle dopaminerger Innervation der Speicheldrüsen dar (ELIA ET AL., 1994; BAUMANN ET AL., 2002). Der Neurotransmitter des SN2-Neurons ist noch nicht zweifelsfrei bekannt. Es scheint jedoch nicht Serotonin zu sein (GIFFORD ET AL., 1991; DAVIS, 1987), wie bei der Heuschrecke *Locusta migratoria* (ALI ET AL., 1993; ALI, 1997), sondern eher GABA wie im SN2-Neuron der Heuschrecke *Schistocerca gregaria* (WATKINS & BURROWS, 1989). Jeder Speichelgangnerv von *Periplaneta* enthält zwei Axone mit einem Durchmesser von 5-7 µm. Weiterhin befinden sich mehrere feine serotonerge Nervenfasern im Speichelgangnerven (WHITEHEAD, 1971; DAVIS, 1985; BAUMANN ET AL., 2002). Die Speicheldrüsen werden auch über die paarigen Speichelnerven, die ihren Ursprung im Ösophagusnerven des stomatogastrischen Nervensystems haben, serotonerg innerviert (WILLEY, 1961; BAUMANN ET AL., 2002). Die Drüsenacini sind von einem Netzwerk dopaminerger und serotonerger variköser Fasern umgeben (BAUMANN ET AL., 2002, 2004). Dopaminerge Fasern befinden sich nur an der Oberfläche der Acini (in räumlicher Nähe zu den P-Zellen) und entlang des gesamten Gangsystems. Der innere Teil der Drüsenläppchen, der die C-Zellen enthält, ist frei von dopaminerger Innervation. Ca. 11% der Synapsin-positiven Strukturen werden auch mit einem Antikörper gegen Tyrosinhydroxylase markiert. Diese Endigungen befinden sich ausschließlich auf der Oberfläche der Acini in der Nähe der P-Zellen und sind vermutlich die Orte, aus denen Dopamin freigesetzt wird. Serotonerge Fasern bilden ebenfalls ein Netzwerk an der Oberfläche der Acini, aber sie projizieren auch tief in die Acini hinein. In ca. 89% der Synapsin-positiven Strukturen konnte Serotonin co-lokalisiert werden. Diese Ergebnisse zeigen, dass die P-Zellen sowohl von serotonergen als auch von dopaminergen Fasern innerviert werden. Im Gegensatz dazu haben C-Zellen vermutlich nur mit serotonergen Fasern synaptischen Kontakt. Dopaminerge und serotonerge Synapsin-positive Strukturen befinden sich auch im Epithel der Ausführgänge. Während erstere über das gesamte Gangsystem nachgewiesen werden konnten, fanden sich serotonerge Fasern nur in Acini-nahen Gangabschnitten.

7.2 Physiologische Untersuchungen zur Beteiligung von sekundären Botenstoffen an der Proteinsekretion durch die C-Zellen

Unser Wissen über die Signalkaskaden, welche die Stimuli „Dopamin“ und „Serotonin“ mit den grundlegenden Teilmechanismen der Speichelsekretion, das sind „Elektrolyt- und Wassersekretion“, „Proteinsekretion“ bzw. „Modifikation des Primärspeichels“, verbinden, ist noch sehr lückenhaft (**WALZ ET AL., 2006**). Sekretionsmessungen lieferten erste Informationen darüber, welche sekundären Botenstoffe an der Proteinsekretion durch die C-Zellen beteiligt sind (**RIETDORF ET AL., 2005**). Die Proteinsekretion isolierter Speicheldrüsen wird durch Serotonin dosisabhängig stimuliert. Der Schwellenwert (30 nM) und der EC_{50}-Wert (1,5 µM) für Serotonin sind nahezu identisch mit den Serotonin-Konzentrationen, die für die Flüssigkeitssekretion aus Speicheldrüsen bestimmt wurden (JUST & WALZ, 1996). Dadurch

wird möglicherweise sichergestellt, dass das durch Stimulation mit Serotonin freigesetzte Protein effektiv durch die Speichelgänge transportiert wird. Wir konnten zeigen, dass die Serotonin-induzierte Proteinsekretion durch eine Erhöhung der $[cAMP]_i$ vermittelt wird (**RIETDORF ET AL., 2005**). Es wurden verschiedene experimentelle Ansätze gewählt, um die $[cAMP]_i$ in den C-Zellen isolierter Schaben-Speicheldrüsen zu erhöhen: 1) Direkte Applikation von Dibutyryl-cAMP, 2) Aktivierung der Adenylyl-Zyklasen mit Forskolin, 3) Hemmung der Phosphodiesterasen mit IBMX sowie 4) Kombinationen dieser Behandlungen. Bei allen Ansätzen konnte Proteinsekretion nachgewiesen werden (**RIETDORF ET AL., 2005**). Im Vergleich zu cAMP scheint Ca^{2+} lediglich als Co-Agonist zu wirken und die Rate der Proteinsekretion weiter zu steigern. Die experimentelle Erhöhung der $[Ca^{2+}]_i$ mit Ionomycin allein ist nicht ausreichend, um eine Proteinsekretionsrate zu induzieren, wie sie nach Serotonin-Stimulation beobachtet wird (**RIETDORF ET AL., 2005**).

7.3 Pharmakologische Eigenschaften der Dopamin-Rezeptoren auf den Speicheldrüsen der Schabe

Mittels Sekretionsmessungen haben wir die pharmakologischen Eigenschaften der Dopamin-induzierten sekretorischen Speicheldrüsenaktivität untersucht (**MARG ET AL., 2004; WALZ ET AL., 2006**). Die Wirkung von Dopamin kann durch den nicht-selektiven Agonisten 6,7-ADTN und, allerdings weniger effektiv, durch den Dopamin-D1-Rezeptor-selektiven Agonisten Chloro-APB imitiert werden (siehe Abb. 13). Der D1-Rezeptor-selektive Agonist SKF 38393 und der D2-Rezeptor-selektive Agonist R(-)-TNPA sind dagegen unwirksam (siehe Abb. 13). Im Vergleich zur Dopamin-induzierten Reaktion löst R(+)-Lisurid eine zeitverzögerte und reduzierte Flüssigkeitssekretion aus. Allerdings hält die sekretorische Aktivität auch nach dem Auswaschen des Lisurids an. Die Wirkung von Dopamin kann durch die Antagonisten *cis*(Z)-Flupenthixol (nicht-selektiv), Chlorpromazin (Dopamin-D2-Rezeptor-selektiv) und S(+)-Butaclamol (nicht-selektiv) blockiert werden (siehe Abb. 14). Zusammenfassend zeigen diese Ergebnisse, dass sich die pharmakologischen Eigenschaften der Dopamin-Rezeptoren der Schabe deutlich von denen der Wirbeltiere unterscheiden (**MARG ET AL., 2004; WALZ ET AL., 2006**). Aufgrund dieser pharmakologischen Daten ist es daher noch nicht möglich zu entscheiden, ob die Dopamin-Rezeptoren, die auf der Speicheldrüse von *Periplaneta* exprimiert werden, zur Unterfamilie der D1- oder der D2-Rezeptoren gehören.

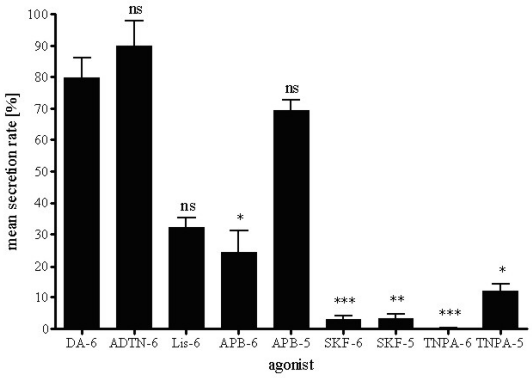

Abbildung 13: Mittlere Sekretionsraten isolierter Speicheldrüsen nach Stimulation mit verschiedenen Dopamin-Rezeptor-Agonisten (aus: **MARG ET AL., 2004**). Das Balkendiagramm zeigt die mittleren Sekretionsraten nach Stimulation mit 1 µM Dopamin (DA-6), 1 µM 6,7-ADTN (ADTN-6), 1 µM Lisurid (Lis-6), 1 µM Chloro-APB (APB-6), 10 µM Chloro-APB (APB-5), 1 µM SKF 38393 (SKF-6), 10 µM SKF 38393 (SKF-5), 1 µM R(-)-TNPA (TNPA-6) und 10 µM R(-)-TNPA (TNPA-5). Die Sekretionsraten nach der Stimulation mit Agonisten wurden mit den Raten nach Stimulation mit 1 µM Dopamin statistisch verglichen. (*Dunn's multiple comparison test*; ns $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

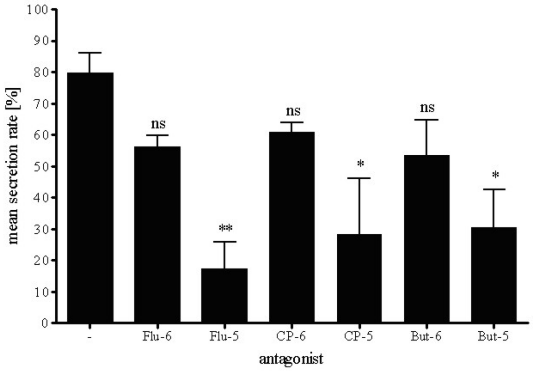

Abbildung 14: Hemmung der Dopamin-induzierten Sekretion durch Dopamin-Rezeptor-Antagonisten (aus: **MARG ET AL., 2004**). Das Balkendiagramm zeigt die mittleren Dopamin-induzierten Sekretionsraten in der Abwesenheit von Antagonisten (-) und in der Gegenwart von 1 µM Flupenthixol (Flu-6), 10 µM Flupenthixol (Flu-5), 1 µM Chlorpromazin (CP-6), 10 µM Chlorpromazin (CP-5), 1 µM Butaclamol (But-6), und 10 µM Butaclamol (But-5). Alle drei Dopamin-Rezeptor-Antagonisten verursachen eine dosisabhängige Unterdrückung der Dopamin-induzierten Sekretion. Die Sekretionsraten in der Gegenwart von Antagonisten wurden mit der Sekretionsrate in der Abwesenheit von Antagonisten statistisch verglichen (*Dunn's multiple comparison test*; ns $P > 0.05$; * $P < 0.05$; ** $P < 0.01$).

7.4 Molekulare Identifizierung biogener Amin-Rezeptoren der Schabe

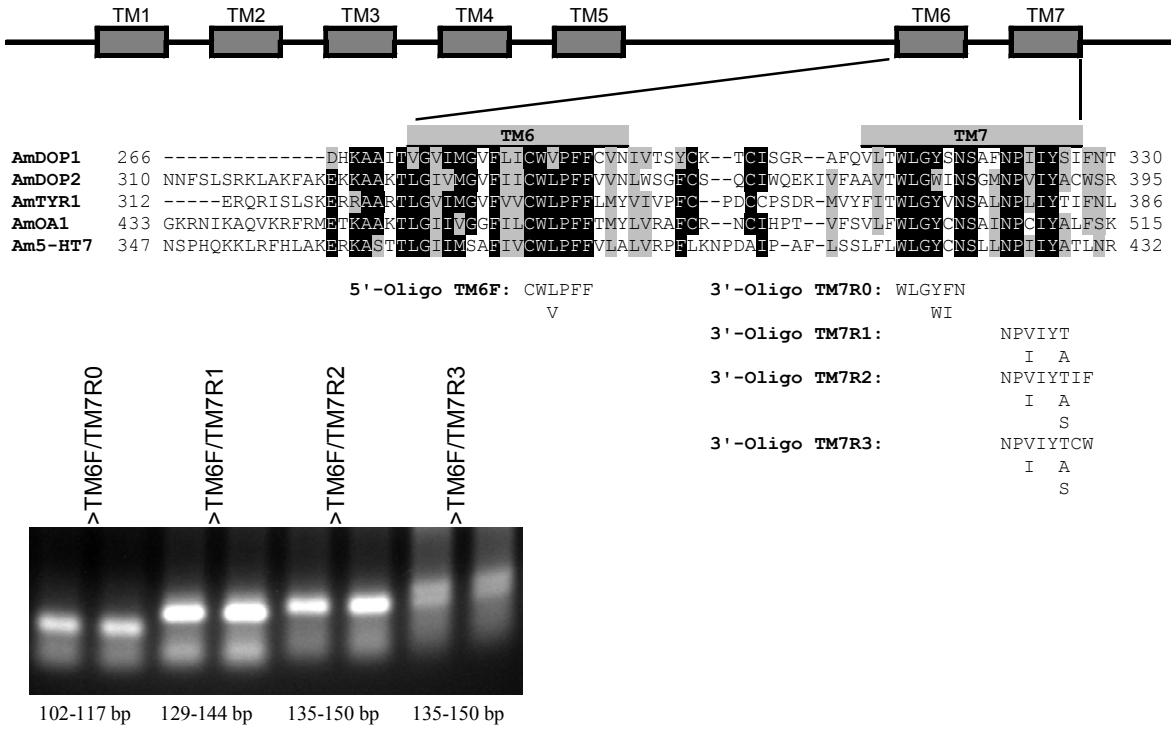

Abbildung 15: Molekulare Analyse von biogenen Amin-Rezeptoren der Schabe *Periplaneta* (aus: **WALZ ET AL., 2006**). Ein Amin-Rezeptor durchspannt die Plasmamembran typischerweise siebenmal.. Diese Transmembran-Domänen (TM 1-7) sind als Zylinder dargestellt (oberer Teil der Abb.). Aminosäuresequenz-Alignment von fünf biogenen Amin-Rezeptoren der Honigbiene (*Apis mellifera*, Am). Aus Gründen der Übersichtlichkeit zeigt der mittlere Teil der Abb. nur den Sequenzvergleich von TM6 bis TM7: AmDOP1 = Dopamin-Rezeptor 1 (BLENAU ET AL., 1998), AmDOP2 = Dopamin-Rezeptor 2 (HUMPHRIES ET AL., 2003), AmTYR1 = Tyramin-Rezeptor 1 (**BLENAU ET AL., 2000**), AmOA1 = Octopamin-Rezeptor 1 (**GROHMANN ET AL., 2003**), Am5-HT_7 = 5-HT_7-Rezeptor (**SCHLENSTEDT ET AL., EINGEREICHT**). Auf der Grundlage der unter dem Alignment dargestellten, konservierten Aminosäuresequenz-Motive wurden degenerierte Oligonukleotide für PCR-Experimente synthetisiert. Der untere Teil der Abbildung zeigt PCR-Produkte, die mit einer *Periplaneta*-Gehirn-cDNA-Bibliothek als „template" amplifiziert wurden.

Wir haben damit begonnen, biogene Amin-Rezeptoren der Schabe *Periplaneta americana* molekular zu identifizieren (**WALZ ET AL., 2006**). Wir benutzen eine oligo-dT-geprimte cDNA-Bibliothek aus Schabengehirnen in λ-ZAPII als „template" für PCR-Experimente mit degenerierten Primern, um Rezeptor-cDNAs zu amplifizieren. Erfolgreich waren Primer-kombinationen, die hochkonservierte Sequenzen innerhalb der TM6 (TM6F) und TM7

(TM7R0, TM7R1, TM7R2 und TM7R3) abdecken (siehe Abb. 15; **WALZ ET AL., 2006**). Die PCR-Fragmente sind 102 - 150 bp groß (siehe Abb. 15). Die Fragmente wurden in den Vektor pGEM-T ligiert und anschließend sequenziert. Die Sequenzanalyse zeigte, dass sie von vier verschiedenen Rezeptoren der Schabe stammen. Abb. 16 zeigt Sequenzvergleiche der abgeleiteten Aminosäuresequenz dieser Schaben-cDNAs (Pea) mit den Aminoäuresequenzen der jeweils am nächsten verwandten biogenen Amin-Rezeptoren aus *Drosophila* (Dm).

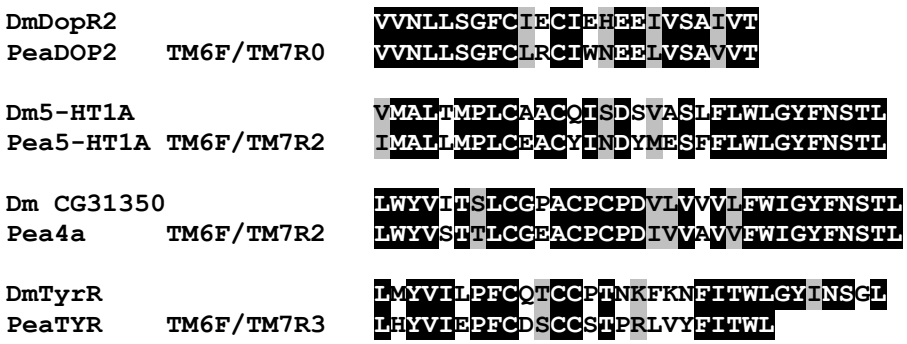

Abbildung 16: Vergleich der abgeleiteten Aminosäuresequenzen von biogenen Amin-Rezeptor-Fragmenten von *Periplaneta* (Pea) and *Drosophila* (Dm) (aus: **WALZ ET AL., 2006**). Identische Aminosäuren sind schwarz unterlegt. Konservierte Aminosäuren sind grau unterlegt. DmDopR2, Dopamin-Rezeptor 2 (HAN ET AL., 1996); Dm5-HT1A, Serotonin-Rezeptor 1A (SAUDOU ET AL., 1992); Dm CG31350, potentieller biogener Amin-Rezeptor (BRODY & CRAVCHIK, 2000), DmTyrR, Tyramin-Rezeptor (SAUDOU ET AL., 1990); TM6F, TM7R0, TM7R2, TM7R3, zur Amplifikation der entsprechenden Fragmente aus *Periplaneta* eingesetzte degenerierte Primer.

Die isolierten cDNA Fragmente kodieren für einen potentiellen Dopamin-, einen potentiellen Serotonin- und einen potentiellen Tyramin-Rezeptor der Schabe. Der Klon Pea4a kann zur Zeit noch nicht eingeordnet werden, weil auch der orthologe Rezeptor aus *Drosophila* (CG31350) bisher nicht funktionell charakterisiert wurde (**WALZ ET AL., 2006**). Die Sequenzinformationen der Partialklone wurden genutzt, um spezifische Primer für 5'- und 3'-RACE-PCR-Experimente zu synthetisieren. Für den Dopamin-, den Tyramin- und den Serotonin-Rezeptor sind die cDNAs inzwischen nahezu vollständig kloniert (C. KRACH, B. TROPPMANN, A. BAUMANN, B. WALZ & W. BLENAU, UNVERÖFFENTLICHT). Durch RT-PCR-Experimente konnten wir zeigen, dass die mRNAs dieser Rezeptoren nicht nur im Gehirn der Schabe, sondern auch in verschiedenen anderen Organen, z.B. in den Speicheldrüsen, exprimiert werden (C. KRACH, B. TROPPMANN, A. BAUMANN, B. WALZ & W. BLENAU, UNVERÖFFENTLICHT).

8. Charakterisierung des *ebony*-Gens der Schabe *Periplaneta americana*

Im Gegensatz zum Wissensstand über die Signalkaskaden, die durch biogene Amine aktiviert werden, ist nur sehr wenig über die Mechanismen zur Beendigung der Wirkung von biogenen Aminen bekannt. Biogene Amine werden inaktiviert durch: (1) „Re-uptake“ in die freisetzende Zelle durch Amin-spezifische Transporter und (2) Abbau durch Enzyme und/oder Konjugatbildung. Eine Familie Na^+/Cl^--abhängiger Transporter mit funktionellen Eigenschaften, die den Amin-Transportern der Wirbeltiere ähneln, ist für Insekten beschrieben worden (CAVENEY & DONLY, 2002). Die Enzyme Monoamin-Oxidase (MAO) und Catecholamin-O-Methyltransferase (COMT), die bei Säugetieren biogene Amine metabolisieren, spielen im Nervensystem von Insekten, wenn überhaupt, nur eine untergeordnete Rolle (SLOLEY, 2004). Hier wird die Inaktivierung der biogenen Amine durch alternative Stoffwechselwege, wie z.B. N-Methylierung und N-Acetylierung, erreicht (WRIGHT, 1987; ROEDER ET AL., 2003). Weiterhin stellt die β-Alanyl-Konjugation biogener Amine durch Ebony-Proteine einen neuen Stoffwechselweg zur Inaktivierung dar (PÉREZ ET AL., 2002, 2004; RICHARDT ET AL., 2003). Mutationen im *ebony*-Gen von *Drosophila* verursachen eine Reihe von Phänotypen, von ebenholzfarbener Kutikula (BREHME, 1941) bis hin zu Verhaltensdefekten (JACOBS, 1978; NEWBY & JACKSON, 1991; BAIER ET AL., 2002). Neuere Untersuchungen haben gezeigt, dass das Ebony-Protein β-Alanin und verschiedene biogene Amine zu β-Alanyl-Konjugaten der biogenen Amine umsetzt (HOVEMANN ET AL., 1998; RICHARDT ET AL., 2003).

Wir haben einen vollständigen *ebony*-cDNA-Klon (Pea*ebony*) aus einer Schabengehirn-cDNA-Bibliothek isoliert (**BLENAU & BAUMANN, 2005**). Die abgeleitete Aminosäuresequenz (PeaEbony) besteht aus 860 Resten. Deutliche Sequenzähnlichkeit besteht zu Ebony-Proteinen von *Anopheles gambiae*, *Drosophila melanogaster* und *Apis mellifera*. PeaEbony besitzt ebenfalls große Sequenzähnlichkeit zu einer Familie mikrobieller, nicht-ribosomaler Peptid-Synthasen (NRPS). Auffällig ist, dass Sequenzmotive innerhalb der reaktiven Zentren, die für NRPS genau bekannt sind (MARAHIEL ET AL., 1997), in PeaEbony konserviert sind (siehe Abb. 17). Insbesondere ein hochkonservierter Serin-Rest innerhalb der Domäne für die Thioester-Bildung von NRPS, der für die Bindung des Co-Faktors 4-Phosphopantetheinyl von Bedeutung ist (LAMBALOT ET AL., 1996), ist auch in PeaEbony (S_{597}, **BLENAU & BAUMANN, 2005**) und *Drosophila*-Ebony (S_{611}, RICHARDT ET AL, 2003) konserviert. Interessanterweise zeigen die C-terminalen 230 Aminosäurereste von PeaEbony keinerlei Homologie zu NRPS. Möglicherweise dient dieser Bereich dazu, das Substrat (= ein biogenes Amin) zu binden (HOVEMANN ET AL., 1998; RICHARDT ET AL., 2003). HOVEMANN und Mitarbeiter haben das in Abb. 18 dargestellte Modell für den Reaktionsmechanismus postuliert (HOVEMANN ET AL., 1998) und experimentell nachgewiesen (RICHARDT ET AL., 2003). Im ersten Schritt wird das durch die Adenylierungs-Domäne gebundene β-Alanin durch ATP aktiviert, dabei entsteht β-Alanyl-Adenylat. Im zweiten Schritt wird das β-Alanyl-Adenylat intramolekular auf eine 4’-Phosphopantetheinyl-Gruppe übertragen, die an einen Serin-Rest gebunden ist. Ein Thioester wird gebildet und cAMP wird freigesetzt. Das β-Alanin reagiert dann mit der primären Aminogruppe eines biogenen Amins (z.B. Dopamin) und es entsteht das β-Alanyl-Konjugat des biogenen Amins (z.B. β-Alanyl-Dopamin).

domain	core		
adenylation	A1	consensus sequence	L (TS) Y x E L
		PeaEbony	L V V A L L
	A2	consensus sequence	L K A G x A Y L (VL) P (LI) D
		PeaEbony	W K A G A A Y L - P L D
	A3	consensus sequence	L A Y x x Y T S G (ST) T G x P K G
		PeaEbony	L A L V L Y T S G S T G V P K G
	A4	consensus sequence	F D x S
		PeaEbony	F V D S
	A5	consensus sequence	N x Y G P T E
		PeaEbony	N F Y G S T E
	A6	consensus sequence	G E L x I x G x G (VL) A R G Y L
		PeaEbony	G E L Y V S G L N L A Q G Y V
	A7	consensus sequence	Y (RK) T G D L
		PeaEbony	Y R T G D F
	A8	consensus sequence	G R x D x Q V K I R G x R I E L G E I E
		PeaEbony	G R T D S Q V K I R G H R V D L A E V E
	A9	consensus sequence	L P x Y M (IV) P
		PeaEbony	L A A Y M L P
	A10	consensus sequence	N G K (VL) D R
		PeaEbony	N G K T D R
thiolation	T	consensus sequence	D x F F x x L G G (HD) S (LI)
		PeaEbony	Q A N F Y E L G G N S L

Abbildung 17: Sequenzvergleich von Konsensussequenz-Motiven (consensus sequence) für die Adenylierung (adenylation) und die Thioester-Bildung (thioester formation) von nicht-ribosomalen Peptid-Synthasen (NRPS; MARAHIEL ET AL., 1997, RICHARDT ET AL., 2003) und PeaEbony (aus: **BLENAU & BAUMANN, 2005**). Identische Aminosäuren sind schwarz unterlegt. Konservierte Aminosäuren sind grau unterlegt.

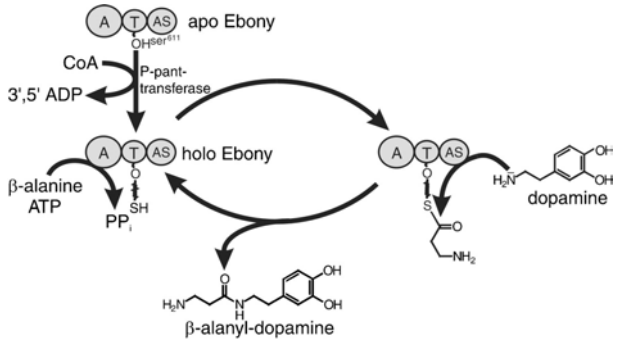

Abbildung 18: (aus: RICHARDT ET AL., 2003). Vorgeschlagener Mechanismus für die Ebony-katalysierte Bindung von Dopamin an β-Alanin. Für Details: siehe Text.

Die Pea*ebony* mRNA wird im Gehirn stark exprimiert und ist auch in den Speicheldrüsen der Schabe nachweisbar (**BLENAU & BAUMANN, 2005**). Wir nehmen an, dass Ebony in der Schabe an der Inaktivierung biogener Amine durch β-Alanyl-Konjugation beteiligt ist. In den Speicheldrüsen könnte Ebony sowohl Dopamin als auch Serotonin inaktivieren.

9. Zusammenfassung und Ausblick

Biogene Amine sind wichtige Vermittler und Regulatoren der neuronalen Signalverarbeitung im zentralen und peripheren Nervensystem von Insekten. Eine wichtige Voraussetzung für das Verständnis der physiologischen Bedeutung aminerger Signalsysteme ist die molekulare Charakterisierung der entsprechenden Amin-Rezeptoren und ihrer nachgeschalteten Reaktionspartner. Die Anwendung molekularbiologischer Methoden hat unser Wissen über die Rezeptorpolypeptide stark erweitert. Wie bei den Wirbeltieren vermitteln mehrere Rezeptorsubtypen die Wirkungen biogener Amine bei Insekten. Mit wenigen Ausnahmen (Histamin-gesteuerte Ionenkanäle) gehören die bisher identifizierten biogenen Amin-Rezeptoren der Insekten zur großen Familie der G-Protein-gekoppelten Rezeptoren. Generell führt die Aktivierung heterolog exprimierter Rezeptorpolypeptide zur Ansteuerung der gleichen intrazellulären Botenstoffwege wie die Aktivierung nativer Rezeptoren *in vivo*. Einige Rezeptorsubtyen scheinen jedoch immer noch zu „fehlen“, da nicht alle *in vivo* beschriebenen pharmakologischen und Signal-Eigenschaften von biogenen Amin-Rezeptoren durch bisher klonierte Rezeptoren abgedeckt werden. Mit der Verfügbarkeit kompletter genomischer Sequenzen einiger „Modellinsekten“ (*Drosophila melanogaster, Anopheles gambiae, Apis mellifera*) und fortgesetzter molekularbiologischer Forschungsarbeit werden diese Lücken jedoch in der nahen Zukunft geschlossen werden (BRODY & CRAVCHIK, 2000; VANDEN BROECK, 2001; **BLENAU & BAUMANN, 2001**; HILL ET AL., 2002; **BLENAU, 2005**). Die Kombination der experimentellen Vorteile, die verschiedene Insektenarten bieten (z.B. *Drososphila* mit ihrem genetischen Potential, die Honigbiene zum Studium der neuronalen und biochemischen Grundlagen von Lernprozessen und die Speicheldrüsen von *Periplaneta* für das Studium der zellulären Wirkungen biogener Amine), wird mit Sicherheit unser Verständnis zur Bedeutung aminerger Signaltransduktionswege bei Entwicklungs-, physiologischen und Verhaltensprozessen verbessern.

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- BAUMANN, A., **BLENAU, W.** AND ERBER, J. (2003) Biogenic amines. In: V. H. RESH AND R. T. CARDÉ (EDS.) *Encyclopedia of Insects*. Academic Press, San Diego, 91-94.
- **BLENAU, W.** AND BAUMANN, A. (2003) Aminergic signal transduction in invertebrates: focus on tyramine and octopamine receptors. *Recent Research Developments in Neurochemistry* **6**, 225-240.
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Molecular and Pharmacological Properties of Insect Biogenic Amine Receptors: Lessons From *Drosophila melanogaster* and *Apis mellifera*

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In the central nervous system (CNS) of both vertebrates and invertebrates, biogenic amines are important neuroactive molecules. Physiologically, they can act as neurotransmitters, neuromodulators, or neurohormones. Biogenic amines control and regulate various vital functions including circadian rhythms, endocrine secretion, cardiovascular control, emotions, as well as learning and memory. In insects, amines like dopamine, tyramine, octopamine, serotonin, and histamine exert their effects by binding to specific membrane proteins that primarily belong to the superfamily of G protein–coupled receptors. Especially in *Drosophila melanogaster* and *Apis mellifera* considerable progress has been achieved during the last few years towards the understanding of the functional role of these receptors and their intracellular signaling systems. In this review, the present knowledge on the biochemical, molecular, and pharmacological properties of biogenic amine receptors from *Drosophila* and *Apis* will be summarized. Arch. Insect Biochem. Physiol. 48:13–38, 2001. **© 2001 Wiley-Liss, Inc.**

Key words: dopamine; G protein-coupled receptors; histamine; honeybee; octopamine; serotonin; tyramine

INTRODUCTION

Communication between individual neurons as well as between neurons and non-neuronal target cells is mediated by unique electro-chemical signaling pathways. Action potentials lead to the release of chemical "messengers" from specialized cell regions of the excited neuron. These messengers include small organic or peptidergic compounds, which either act as neurotransmitters, neuromodulators, or neurohormones. Some compounds may even have overlapping properties, acting as neurotransmitters in locally confined interneuronal signaling or as neurohormones when released into the blood or haemolymph and transported via the circulatory system to their target tissues in the body. The messengers are rec-

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ognized by specific receptors on the surface membrane of the target cell. Interaction between the messenger and its receptor translates the chemical signal into a specific electrical or biochemical response of the target cell.

Although the structural features of neuroactive substances are quite diverse, the cellular responses evoked by these compounds are generally mediated by members of only two large gene families encoding different types of membrane receptors. Binding of the messengers to ionotropic receptors (ligand-gated ion channels) leads to the opening of the channel pore and causes either excitation or inhibition of the target cell. In contrast to ion channel activation, binding of messengers to metabotropic receptors (G protein-coupled receptors; GPCRs) leads to slower cellular responses. Activated GPCRs transmit the signal to intracellular trimeric GTP-binding (G) proteins (see Structural Properties of Biogenic Amine Receptors). Once activated, the G proteins either stimulate or inhibit specific target proteins. This causes changes in the concentration of intracellular "second messengers," e.g., cyclic nucleotides (cAMP, cGMP), inositol 1,4,5-trisphosphate (IP_3), and diacylglycerol (DAG) (see Signaling Pathways Activated by Biogenic Amine Receptors). Finally, second messenger-dependent enzymes are activated and transiently modify the functional properties of various cytosolic, membrane-bound, or nuclear proteins. G protein subunits may also regulate ion channel activity directly (Hille, 1994; Clapham and Neer, 1997; Schneider et al., 1997).

One important group of messenger substances that primarily bind to GPCRs are the biogenic amines (for a review see Vernier et al., 1995). These molecules are synthesized from amino acids in single to multistep reactions (see Biosynthesis of Biogenic Amines), and are found in both vertebrates and invertebrates (see Table 1). In the central nervous system (CNS), biogenic amines control and modulate various functions, including cardiovascular homeostasis, circadian rhythms, emotional states, endocrine secretion, sexual behavior, thermoregulation, as well as learning and memory. In humans, the etiology of several neural diseases has been linked to impaired biogenic amine signaling. Much effort has been given to evaluating the molecular and functional properties of biogenic amine receptors and their downstream reaction partners in order to identify potential targets for the pharmacological treatment of such diseases. In recent years, considerable progress has been made in unraveling the physiological role of biogenic amines and their receptors in invertebrates. The aim of this review is to summarize the molecular, pharmacological, and functional properties of insect biogenic amine receptors. Since our own work is focused on the molecular and pharmacological characterization of receptors from the fruitfly (*Drosophila melanogaster*) and the honeybee (*Apis mellifera*), we will concentrate primarily on these two species.

TABLE 1. List of the Major Biogenic Amines Identified in Vertebrates and Invertebrates

Vertebrates	Invertebrates
Dopamine	Dopamine
Norepinephrine	Tyramine
Epinephrine	Octopamine
Serotonin	Serotonin
Histamine	Histamine

BIOSYNTHESIS OF BIOGENIC AMINES

In both vertebrates and invertebrates, the group of biogenic amine messengers consists of five members (see Table 1). In addition to molecules shared by both phylogenetic groups (dopamine, serotonin, histamine), some biogenic amines seem to be synthesized preferentially in either vertebrates (norepinephrine, epinephrine) or invertebrates (tyramine, octopamine). Biogenic amines are synthesized from three different amino acids and here we will briefly summarize these biosynthetic pathways. Additional information can be found in comprehensive textbooks (Siegel et al., 1998; Hardman et al., 1996) and the citations in this section will consider only publications on *Drosophila*.

Biogenic Amines Derived From Tyrosine

In vertebrates, tyrosine gives rise to the catecholamines dopamine, norepinephrine, and epinephrine (see Fig. 1). Biosynthesis starts with hydroxylation in the *meta*-position of tyrosine and is catalyzed by tyrosine hydroxylase (TH). TH is the rate-limiting enzyme in catecholamine synthesis. In a second step, 3,4-dihydroxy-L-phenylalanine (L-DOPA) is decarboxylated to dopamine. The conversion is mediated by the enzyme DOPA decarboxylase (DDC). In certain vertebrate neurons, dopamine is an intermediate reaction prod-

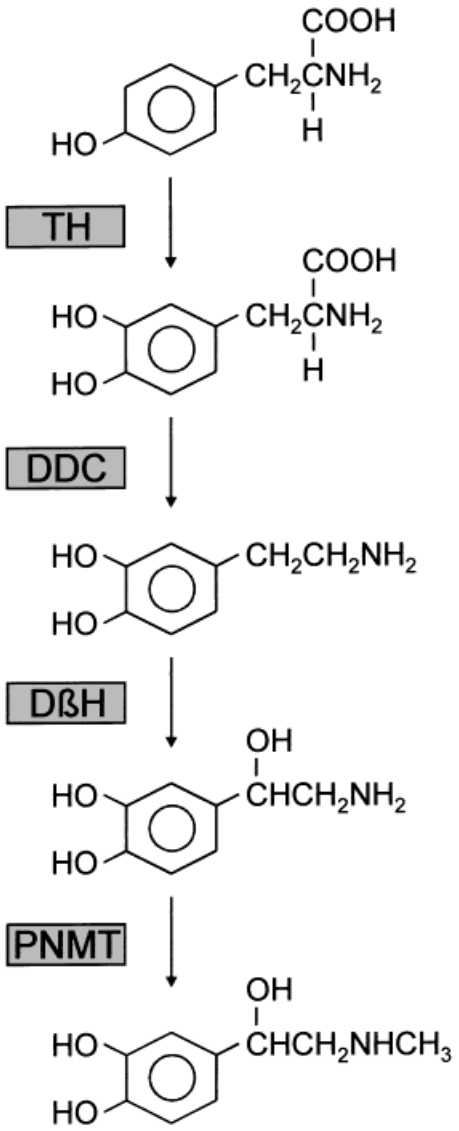

Fig. 1. Biosynthesis of catecholamines. Tyrosine is hydroxylated by tyrosine hydroxylase (TH) to L-DOPA. Decarboxylation of L-DOPA by DOPA decarboxylase (DDC) generates dopamine. Dopamine is hydroxylated by dopamine β-hydroxylase (DβH) to norepinephrine. Norepinephrine is methylated by phenylethanolamine N-methyltransferase (PNMT) to epinephrine.

uct and will be further metabolized. Dopamine β-hydroxylase (DβH) catalyzes the formation of norepinephrine and adds a hydroxyl group to the β-carbon on the side chain of dopamine. But even norepinephrine can be further modified. This is achieved by the enzyme phenylethanolamine N-methyltransferase (PNMT), which adds a methyl group to the nitrogen of norepinephrine, thereby forming the secondary amine epinephrine.

Whereas the identical pathway to synthesize dopamine also exists in invertebrates (Hirsh and Davidson, 1981; Livingstone and Tempel, 1983; Neckameyer and Quinn, 1989; Restifo and White, 1990), norepinephrine and epinephrine have not been unequivocally identified in *Drosophila* (Wright, 1987) although low concentrations have been detected in some other insect species (Brown and Nestler, 1985; Evans, 1980).

In addition to the biosynthesis of dopamine, invertebrates use an alternative biochemical pathway to generate the phenolamines tyramine and octopamine from tyrosine (see Fig. 2). In a first step, tyrosine is decarboxylated to tyramine by tyrosine decarboxylase (TDC; Livingstone and Tempel, 1983). Similar to the conversion of dopamine to norepinephrine, tyramine can also be hydroxylated on the β-carbon of the side chain. This reaction is catalyzed by tyramine β-hydroxylase (TβH) and generates octopamine (Monastirioti et al., 1996). Since octopamine and norepinephrine are chemically very similar, though not identical substances, it has been suggested that the noradrenergic/adrenergic system of vertebrates is functionally substituted by the tyraminergic/octopaminergic system in invertebrates (Evans, 1985, 1993; Roeder, 1999).

Serotonin Is Derived From Tryptophan

In both vertebrates and invertebrates, identical biochemical pathways exist to synthesize the indolamine 5-hydroxytryptamine (5-HT, serotonin) from L-tryptophan (see Fig. 3). In the first and rate-limiting reaction, a hydroxyl group is added to the indole ring in the 5′-position by tryptophan hydroxylase (TRH; Livingstone and Tempel, 1983; Neckameyer and White, 1992). Once synthesized, 5-hydroxytryptophan is decarboxylated by DDC to serotonin (Hirsh and Davidson, 1981; Livingstone and Tempel, 1983). Since DDC also participates in the decarboxylation of L-DOPA to dopamine (see Fig. 1), a defect or loss of function of this enzyme will simultaneously result in a severely impaired production of both dopamine and serotonin. In *Drosophila*, DDC null mutants cause the death of the animal in early developmental stages (Wright, 1987).

Histamine Is Derived From Histidine

A single decarboxylation step converts L-histidine to histamine (see Fig. 4). The reaction is

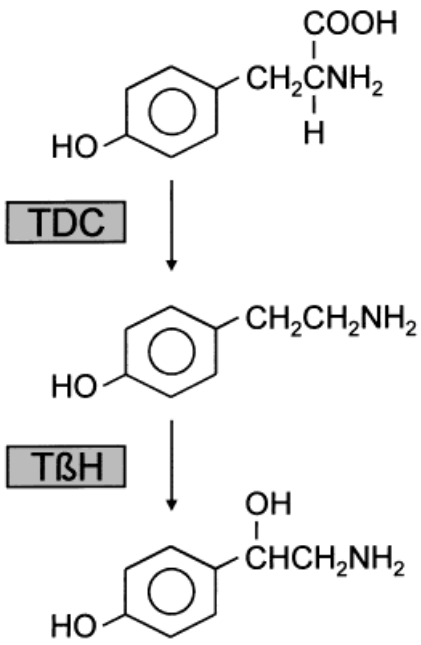

Fig. 2. Biosynthesis of phenolamines. Tyrosine is decarboxylated by tyrosine decarboxylase (TDC) to tyramine which is hydroxylated by tyramine β-hydroxylase (TβH) to octopamine.

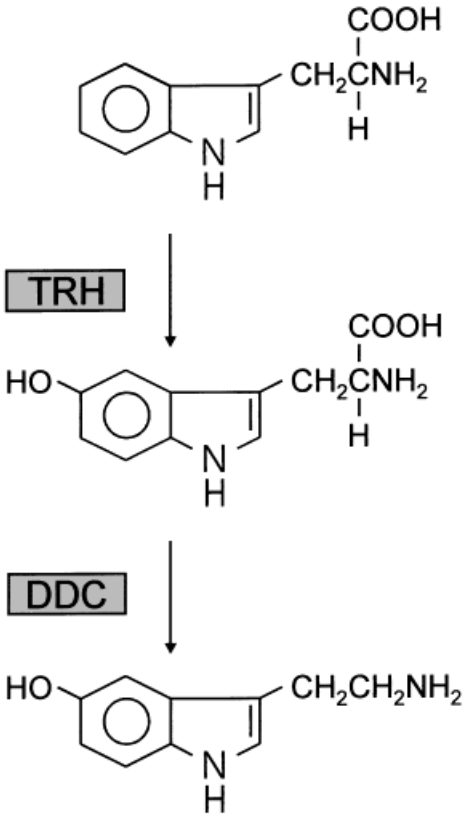

Fig. 3. Biosynthesis of the indolamine serotonin. L-tryptophan is hydroxylated by tryptophan hydroxylase (TRH) to 5-hydroxytryptophan, which is decarboxylated by DOPA decarboxylase (DDC) to serotonin.

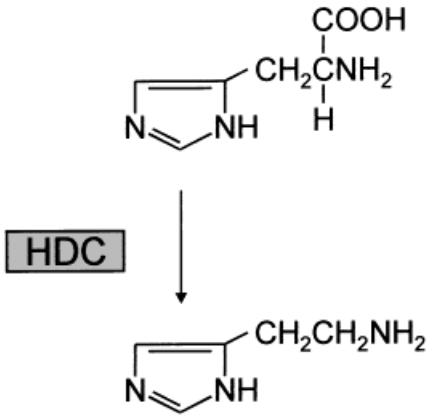

Fig. 4. Biosynthesis of histamine. L-histidine is decarboxylated by histidine decarboxylase (HDC) to histamine.

mediated by the enzyme histidine decarboxylase (HDC; Burg et al., 1993). Histamine has been shown to be the major neurotransmitter released from invertebrate photoreceptors (Hardie, 1989; for reviews see: Nässel, 1991, 1999). In vertebrates, histamine is considered one of the most important mediators of allergy and inflammation. In the vertebrate CNS, however, histamine is synthesized from a small population of neurons located in the posterior hypothalamus. These neurons project to most cerebral areas and have been implicated in hormonal secretion, cardiovascular control, thermoregulation, and memory functions (Schwartz et al., 1991).

STRUCTURAL PROPERTIES OF BIOGENIC AMINE RECEPTORS

Biogenic amine receptors belong predominantly to the superfamily of GPCRs. Within this gene family, biogenic amine receptors fall into the largest subfamily, i.e., rhodopsin-like receptors. All members of this group are integral membrane proteins. Based on crystal structure data (Palczewski et al., 2000) as well as hydropathy profile analyses and phylogenetic comparisons (Baldwin, 1994; Vernier et al., 1995; Baldwin et al., 1997; Valdenaire and Vernier, 1997), these receptors share the common motif of seven transmembrane (TM) domains (see Fig. 5). As type II membrane proteins, the N-terminus is located extracellularly and the C-terminus is located intracellularly. The N-terminus often contains consensus sequence motifs for N-linked glycosylation (Probst et al., 1992; Strader et al., 1995). The membrane-spanning re-

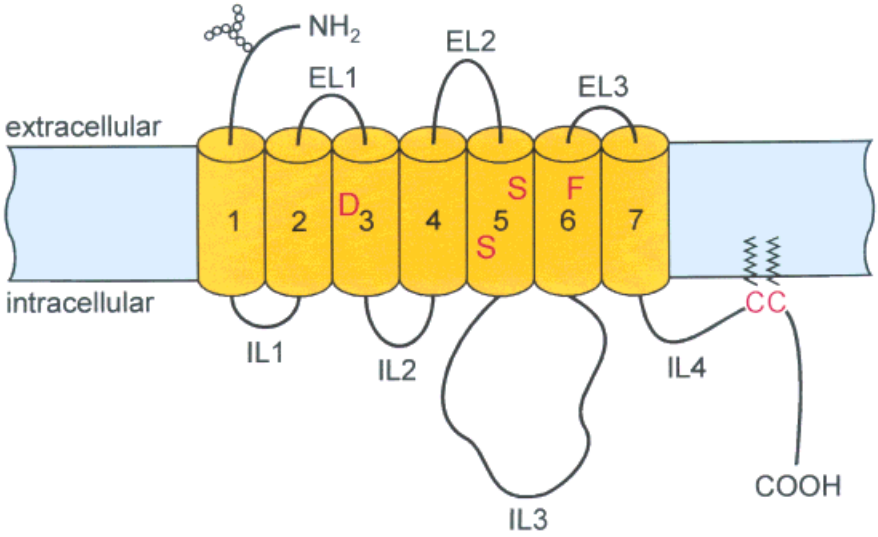

Fig. 5. Topography of a biogenic amine receptor. The polypeptide spans the membrane seven times. These transmembrane regions (TM 1–7) are depicted as cylinders. The N-terminus (NH_2) is located extracellularly and often contains glycosylated residues (small open circles). The C-terminus (COOH) is located intracellularly. The membrane spanning regions are linked by three extracellular loops (EL) that alternate with three intracellular loops (IL). Residues that participate in ligand binding are indicated in grey: an aspartic acid residue (D) in TM3, serine residues (S) in TM5, and a phenylalanine residue (F) in TM6. When the protein is posttranslationally palmitoylated at cysteine residues (C) in the cytoplasmic tail, a fourth intracellular loop (IL4) will be formed.

gions are linked by three extracellular loops (EL) that alternate with three intracellular loops (IL). A pair of cysteine residues in EL2 and TM3 are believed to form a disulfide bridge that contributes to the structural stability and binding properties of these receptors (Noda et al., 1994). Additional cysteine residues in the cytoplasmic tail of the polypeptides are the target of posttranslational palmitoylation. Insertion of these fatty acids into the plasma membrane will create a fourth intracellular loop (IL4) and further stabilize the structure of these receptors (Jin et al., 2000). Activation of the receptors occurs by binding of specific biogenic amines. The interaction between the ligand and its receptor takes place in a binding pocket formed by the TM regions in the plane of the membrane. Specific residues in different TM segments interact with functional groups of the biogenic amines. In particular, an aspartic acid residue (D) in TM3, serine residues (S) in TM5, and a phenylalanine residue (F) in TM6 were shown to determine the ligand binding properties of biogenic amine receptors (Strader et al., 1995; Valdenaire and Vernier, 1997). Once the ligand is tightly bound to its receptor, the receptor's conformation will change. This structural change is then transferred to trimeric G proteins. Residues in close vicinity to the plasma membrane of IL2, 3, and 4 determine the specificity and efficacy of G protein-activation. Receptor mediated signaling, however, will finally be turned off by phosphorylation of serine and threonine residues in the C-terminus and IL3 (Chuang et al., 1996; Palczewski, 1997).

SIGNALING PATHWAYS ACTIVATED BY BIOGENIC AMINE RECEPTORS

A common feature of GPCR activation is the subsequent change of intracellular messenger concentrations. Depending on which type of GPCR is activated, a change in the intracellular concentration of cAMP ($[cAMP]_i$) and/or Ca^{2+} ($[Ca^{2+}]_i$) is most likely to take place. Since these are the most commonly found cellular responses to biogenic amine treatments, they are used to functionally classify receptor subtypes. As a result of GPCR activation, $[cAMP]_i$ can change in two directions: $[cAMP]_i$ levels are either elevated or decreased (see Fig. 6). The cellular response strictly relies on the specificity of interaction between the receptor and the G protein (Gudermann et al., 1996, 1997). When the receptor binds to a G_s-type protein, the activated $G_{\alpha s}$ subunit will interact with

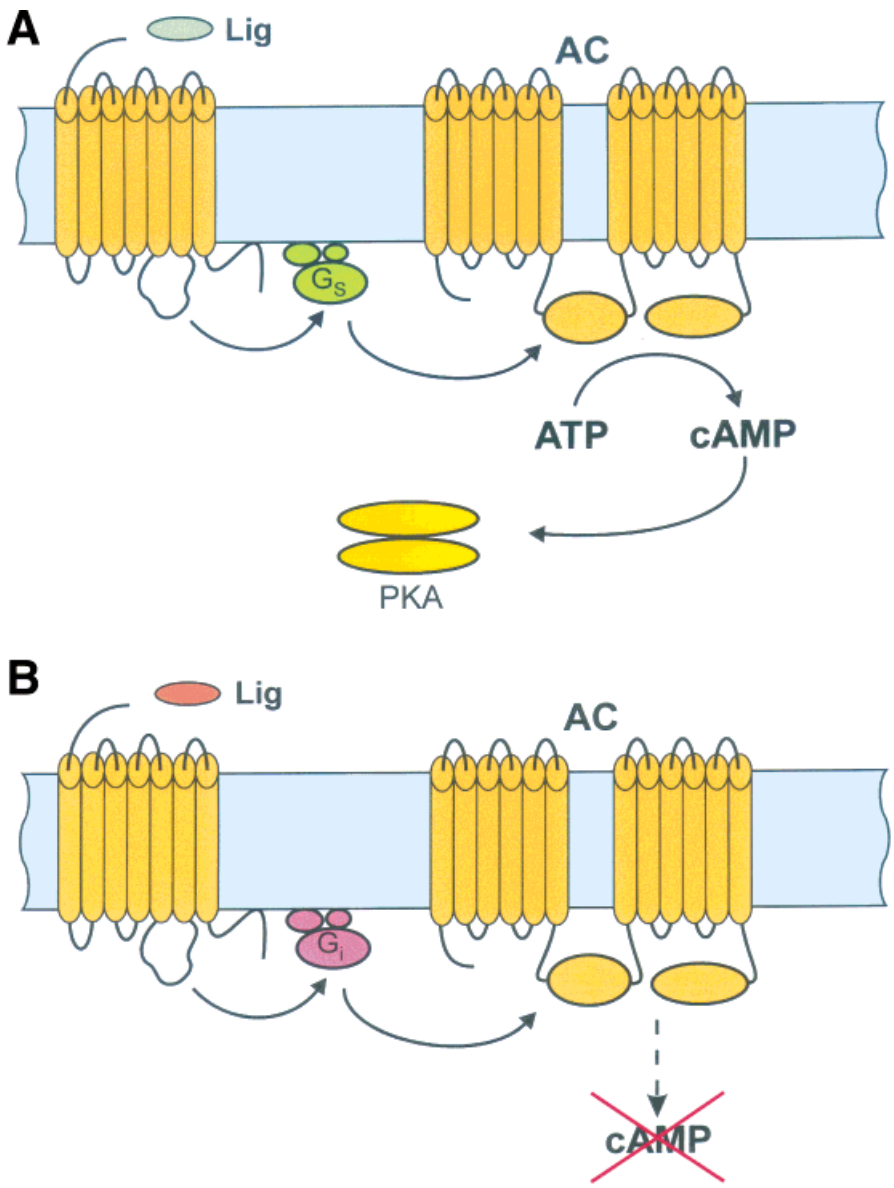

Fig. 6. Biogenic amine receptors coupled to intracellular cAMP signaling pathways. **A:** Biogenic amine receptors are activated by binding of agonists (ligand, Lig). The ligand-bound receptor then activates a stimulatory G protein (G_s), which leads to an increase in the enzymatic activity of adenylyl cyclase (AC). Adenylyl cyclase catalyzes the conversion of ATP to cAMP. As the intracellular concentration of cAMP increases, cAMP-dependent protein kinase (PKA) is activated and phosphorylates different target proteins on serine and threonine residues. **B:** Several biogenic amine receptors are known to inhibit AC activity via inhibitory G proteins (G_i).

adenylyl cyclase (AC) in the plasma membrane. This leads to an increase of cyclase activity and production of cAMP from ATP. The rise in $[cAMP]_i$ will then activate cAMP-dependent protein kinase (protein kinase A, PKA). Phosphorylation of serine and/or threonine residues by PKA modifies the properties of various substrate molecules including cytosolic proteins, ligand-gated and voltage-dependent ion channels, as well as transcription factors, such as CREB, CREM, and ATF-1 (De Cesare et al., 1999). Several biogenic amine receptors are also known to inhibit adenylyl cyclase activity. This effect is mediated by interaction of the receptor with inhibitory G proteins (G_i). Interaction of adenylyl cyclase with activated $G_{\alpha i}$ subunits most likely competes with binding of activated $G_{\alpha s}$ subunits and thereby interferes with cyclase activation.

Another pathway that is activated by several biogenic amine receptors results in a rise of $[Ca^{2+}]_i$ (see Fig. 7). Here, the amine-activated receptor binds to G proteins of the $G_{q/o}$ family (Gudermann et al., 1996, 1997). The activated $G_{\alpha q/o}$ subunits bind to and stimulate phospholipase C (PLC) activity (Rhee and Bae, 1997). The enzyme hydrolyzes a membrane-bound substrate, phosphatidylinositol 4,5-bisphosphate. Cleavage gives rise to two second messengers, IP_3 and DAG. The IP_3 freely diffuses and binds to specific IP_3 receptors on the membrane of intracellular Ca^{2+} stores (endoplasmic reticulum). These receptors are second messenger-gated Ca^{2+} channels. Therefore, after binding of IP_3, the channel pore is opened and Ca^{2+} is released into the cytoplasm. Ca^{2+} ions play a pivotal role in the regulation of many cellular functions by directly controlling enzymatic or ion channel activities. Furthermore, Ca^{2+} can also bind to members of the large family of Ca^{2+}-binding proteins (calmodulin, calbindin, calretinin, etc.) that modulate the activation properties of many effector proteins by protein-protein interaction. Since PLC not only generates IP_3 but also DAG, receptor coupling to $G_{q/o}$ proteins might activate a second signaling pathway in addition to Ca^{2+} release. In contrast to IP_3, DAG remains associated with the membrane, where it activates protein kinase C (PKC). Full enzymatic activity of PKC, however, requires the presence of DAG and Ca^{2+} as well as association of the kinase with the membrane. Similar to PKA (see above), PKC

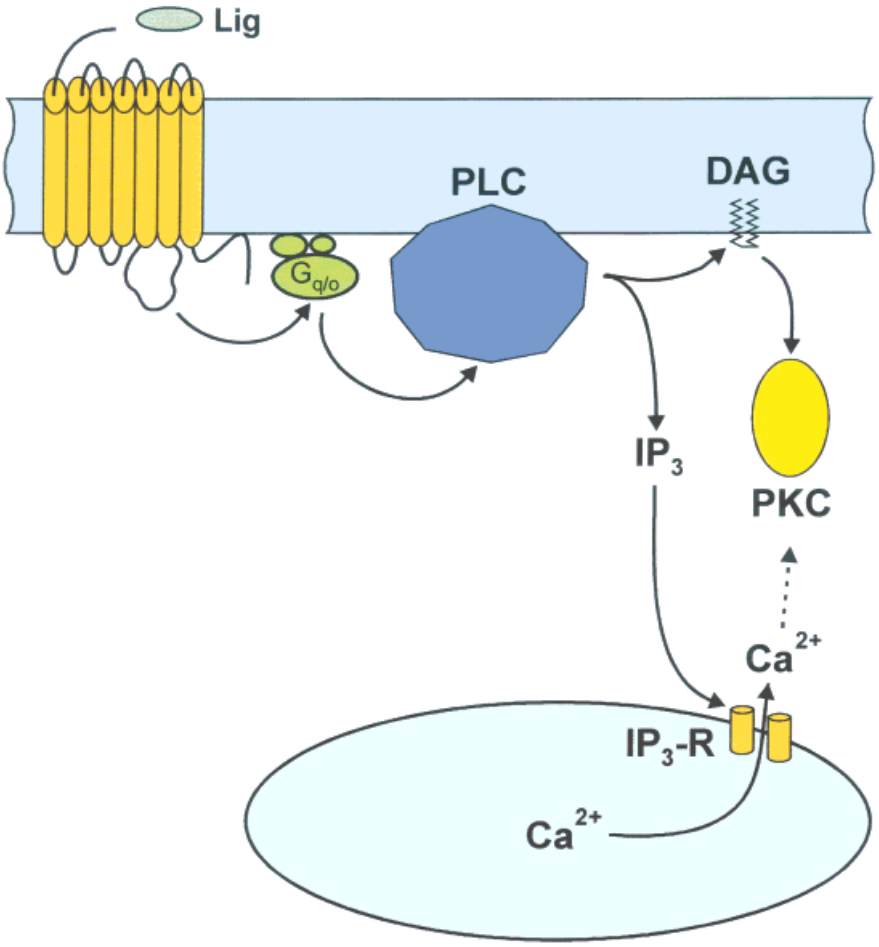

Fig. 7. Biogenic amine receptors coupled to intracellular IP_3/DAG signaling pathways. Agonist (ligand, Lig)-bound biogenic amine receptors activate G proteins of the $G_{q/o}$ family ($G_{q/o}$) that regulate the enzymatic activity of phospholipase C (PLC). This enzyme hydrolyzes phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). Binding of IP_3 to specific receptors (IP_3-R) that form ion channels in the membrane of the endoplasmic reticulum (ER) causes release of Ca^{2+} into the cytoplasm. Both the increase in intracellular Ca^{2+} and the membrane-bound DAG activate protein kinase C (PKC) that phosphorylates different target proteins.

phosphorylates a variety of proteins on serine and threonine residues, which alters the functional properties of these proteins. In summary, GPCR activation generates graded cellular responses depending on the second messenger pathways involved. The different intracellular messenger pathways may also be activated in parallel within the same cell when the respective receptors and coupling partners are present. Such co-activation events potentially lead to either amplifications or diminishments of the cellular responses and provide a cellular basis for "coincidence detection."

DOPAMINE RECEPTORS

The catecholamine dopamine is present in relatively high concentrations in the insect CNS (Mercer et al., 1983; Brown and Nestler, 1985; Fuchs et al., 1989; Harris and Woodring, 1992; Taylor et al., 1992; Kirchhof et al., 1999; Wagener-Hulme et al., 1999; Schulz and Robinson, 1999). Dopaminergic neurons possess widefield arborizations that extend to most areas of the brain suggesting that dopamine plays an important role as a neurotransmitter and/or neuromodulator in insects (Budnik and White, 1988; Nässel and Elekes, 1992; Schäfer and Rehder, 1989; Schürmann et al., 1989; Blenau et al., 1999).

During *Drosophila* development, dopamine levels show four discrete peaks. These coincide with larval molts, pupariation, and adult emergence (Martínez-Ramírez et al., 1992). The analysis of *Drosophila* mutants also suggests a role for dopamine in the terminal differentiation of the nervous system as well as in learning behavior. Mutants that lack the enzyme DDC (*Ddc*, see Biogenic Amines Derived From Tyrosine and Serotonin Is Derived From Tryptophan) and, therefore, are devoid of the biogenic amines dopamine and serotonin, die as early embryos (Tempel et al., 1984). In addition to these null alleles, less severe *Ddc* mutants exist that only have a reduced enzymatic activity of DDC. The greater the impact on DDC activity in these mutants, the worse their performance is in associative learning tests (Tempel et al., 1984). It was also shown that the threshold concentration to sucrose, which elicits the proboscis extension response, is raised ~6-fold in the mutants (Tempel et al., 1984). The learning defect of *Ddc* mutants, however, could not be reproduced in another independent investigation (Tully, 1987). Nevertheless, during larval development *Ddc* mutants show an aberrant pattern of neuronal arborization (Budnik et al., 1989). The extent of branching can be partially restored to wild-type levels by feeding the mutants with dopamine. Inactivation of TH (see Biogenic Amines Derived From Tyrosine) during development results in akinesia, developmental retardation, and decreased fertility of the flies (Neckameyer, 1996). In addition, dopamine-depleted adult males show impaired habituation in experience-dependent courtship paradigms (Neckameyer, 1998).

In the honeybee, the physiological role of different biogenic amines has been examined by behavioral and electrophysiological studies of olfactory brain centers (for a review see: Bicker and Menzel, 1989). Injection of dopamine into the α-lobe of the mushroom bodies, important brain structures for higher order olfactory information processing, alters the electrical responses to olfactory stimuli (Mercer and Erber, 1983) and reduces proboscis extension responses to water vapor (Blenau and Erber, 1998). Dopamine also affects the retrieval of olfactory memories (Mercer and Menzel, 1982; Macmillan and Mercer, 1987; Michelsen, 1988). Since the experimental approach used in these studies may have evoked non-specific responses, an alternative strategy was used by Menzel et al. (1999). Amine levels were first depleted with reserpine. This caused a significant impairment of motor-output patterns. Subsequent injection of dopamine into the brain, restored the reserpine-induced defects in motor patterns, but had no effect on either sensitization or conditioning (Menzel et al., 1999).

In vertebrates, dopamine binds to two subfamilies of dopamine receptors: D1- and D2-(like) receptors (Kebabian and Calne, 1979). These receptors belong to the family of GPCRs and possess different pharmacological and biochemical properties. In humans, D1- and D5-receptors constitute the D1-subfamily and activate adenylyl cyclase, whereas members of the D2-subfamily, i.e., the D2-, D3-, and D4-receptors, either inhibit adenylyl cyclase or couple to different intracellular second messenger systems (for reviews see: Jackson and Westlind-Danielsson, 1994; Missale et al., 1998; Vallone et al., 2000). Benzazepines like R(+)-SCH 23390 bind to D1-like receptors with high

affinity but not to D2-like receptors. In contrast, butyrophenones, like spiperone, bind with high affinity to D2-like but not to D1-like receptors.

Drosophila DmDOP1- and the Honeybee AmDOP1-Receptor

Application of low stringency hybridization protocols led to the cloning of the first dopamine receptor genes from *Drosophila* (Dm*dop1*, Gotzes et al., 1994; *d*DA1, Sugamori et al., 1995). Both genes encode almost identical polypeptides except for a 126–amino acid extension of the N-terminus found only in DmDOP1. This difference most likely is caused by strain-specific nucleotide substitutions and insertions in the *d*DA1-gene, which was cloned from a different *Drosophila* wild-type strain than the Dm*dop1*-gene (Gotzes and Baumann, 1996). Therefore, we will refer to both genes as Dm*dop1*.

Within the putative TMs, DmDOP1 displays high sequence similarity to human dopamine D1- (53.1%) and D5-receptors (51.8%). Amino acid residues that interact with ligands in vertebrate dopamine receptors (Missale et al., 1998) are well conserved in DmDOP1. Functional expression of the Dm*dop1*-gene in human embryonic kidney (HEK 293) cells specifically elicited cAMP production after dopamine application (Gotzes et al., 1994). This response was also evoked by dopamine D1-receptor agonists, e.g., SKF 38393 (Gotzes et al., 1994) and 6,7-ADTN (Sugamori et al., 1995). In contrast to dopamine, stimulation of Dm*dop1*-transfected HEK 293 (Gotzes et al., 1994), COS-7, and *Spodoptera frugiperda* (Sf)9 cells (Sugamori et al., 1995) with the benzazepine SKF 38393 gave rise to only a small increase in $[cAMP]_i$. The production of cAMP was inhibited by dopaminergic antagonists such as S(+)-butaclamol and *cis*(Z)-flupentixol. Most notably, the benzazepine R(+)-SCH 23390, a typical vertebrate D1-receptor antagonist (see above), neither bound with high affinity to DmDOP1 nor was it as potent as butaclamol or flupentixol in inhibiting dopamine-induced cAMP elevation (Gotzes et al., 1994; Sugamori et al., 1995).

The expression pattern of the Dm*dop1*-gene was examined by in situ hybridizations and Northern blot analyses. Receptor mRNA is expressed as a maternal transcript and restricted to apical regions of the cortical peripheral cytoplasm in the embryo (Sugamori et al., 1995). In adult flies, the gene is widely expressed in the CNS (Gotzes et al., 1994). The expression pattern of the receptor gene agrees well with the widespread distribution of dopaminergic nerve fibers determined immunohistochemically.

A homologous gene to Dm*dop1* was recently cloned from honeybee (*Apis mellifera*) brain (Am*dop1*, Blenau et al., 1998). Within the putative TM regions, the deduced amino acid sequence of Am*dop1* (AmDOP1) shares ~75% similarity with vertebrate D1-like receptors and ~93% similarity with DmDOP1. Therefore, the honeybee AmDOP1-receptor most likely is the orthologue of the *Drosophila* DmDOP1-receptor. The pharmacological profile of the heterologously expressed AmDOP1-receptor (Blenau et al., 1998) is very similar to that determined from [^{3}H]LSD binding studies to membrane preparations of honeybee brains (Table 2; Blenau et al., 1995a). [^{3}H]LSD specifically binds to AmDOP1 with a K_D ~5 nM. Of all biogenic amines tested, dopamine was the most potent competitor (K_i = 56 nM). In addition, several synthetic dopaminergic agonists and antagonists also potently displaced [^{3}H]LSD from its binding site on the AmDOP1-receptor (Table 2). Similar to the *Drosophila* DmDOP1-receptor, benzazepines, i.e., SCH 23390 and SKF 38393, which bind to mammalian D1-like receptors with nanomolar affinity, were 200 to >1,000-fold less potent at the AmDOP1-receptor. When either dopamine or 6,7-ADTN was applied to AmDOP1-expressing HEK 293 cells, an increase in $[cAMP]_i$ was observed (Blenau et al., 1998), suggesting that AmDOP1 also belongs to the dopamine D1-like receptor family.

In situ hybridization to tissue sections of adult honeybee brain revealed that Am*dop1* mRNA is expressed in many neurons of the CNS, including neurons of the optic lobes, intrinsic mushroom body neurons, neurons of the antennal lobes, and neurons of the suboesophageal ganglion (Blenau et al., 1998). This widespread distribution suggests that the AmDOP1-receptor is a likely candidate for the processing of higher order sensory information.

Drosophila DAMB/DopR99B-Receptor and the Honeybee AmBAR6-Receptor

A second dopamine receptor gene was cloned from *Drosophila* using polymerase chain reaction

TABLE 2. Pharmacological Properties of Dopamine Receptors in *Apis mellifera

	Apis AmDOP1 in HEK 293: [^{3}H]LSD (K_i [nM])	*Apis* brain homogenate: dopamine-sensitive [^{3}H]LSD binding site (K_i [nM])	*Apis* brain homogenate: [^{3}H]SCH 23390 binding site (K_i [nM])
Biogenic amines			
Dopamine	56	22	30,800
L(–)-Norepinephrine	3,100	—	—
Serotonin	3,600	7,000	548,000
Tyramine	9,900	—	≥1,000,000
DL-Octopamine	110,000	—	892,000
Dopamine receptor agonists			
R(+)-Lisuride	4.3	4.7	—
6,7-ADTN	93	78	—
R(+)-SKF 38393	4,200	—	3,200
Dopamine receptor antagonists			
Chlorpromazine	15	48	208
ciz(*Z*)-Flupentixol	17	150	218
Spiperone	64	—	25,400
S(+)-Butaclamol	77	89	13,800
R(+)-SCH 23390	250	—	9.5
Haloperidol	390	—	—
S(–)-SCH 23388	440	—	—
trans(E)-Flupentixol	650	3,600	—
R(–)-Butaclamol	42,000	>100,000	—

Values for AmDOP1 expressed in HEK 293 cells are from Blenau et al. (1998). Values for the dopamine-sensitive [^{3}H]LSD binding site of the honeybee are from Blenau et al. (1995a) and for the honeybee [^{3}H]SCH 23390 binding site are from Kokay and Mercer (1996). Note the rather low affinity for the AmDOP1-receptor of the benzazepines R(+)-SKF 38393 and R(+)-SCH 23390 and the low affinity of the putative endogenous agonist dopamine for the [^{3}H]SCH 23390 binding site in membrane homogenate of honeybee brains.

(PCR) and single-strand conformation analysis (DAMB, Han et al., 1996; DopR99B, Feng et al., 1996). DAMB and DopR99B encode identical polypeptides except for 27 amino acids at the C-terminus. The difference most likely is caused by incomplete splicing of the DopR99B transcript (Feng et al., 1996; Han et al., 1996). The DAMB/DopR99 cDNA and deduced amino acid sequence, however, clearly differ from the *Drosophila* DmDOP1-receptor (Gotzes et al., 1994).

Functional expression of DAMB in *Drosophila* S2 and HEK 293 cells resulted in an increase in $[cAMP]_i$ upon dopamine application. Since this response was blocked by *cis*(Z)-flupentixol, DAMB is considered an additional member of the subfamily of D1-like dopamine receptors in *Drosophila* (Han et al., 1996). Agonist stimulation of DopR99B expressed in *Xenopus* oocytes led to an increase in $[Ca^{2+}]_i$ as monitored by Ca^{2+}-dependent chloride channel activity (Feng et al., 1996; Reale et al., 1997a). In addition to the calcium response, dopamine application also induced an increase in $[cAMP]_i$ in DopR99B-expressing oocytes (Feng et al., 1996). These results suggest that the DAMB/DopR99B-receptor activates different intracellular signaling pathways in parallel when expressed in *Xenopus* oocytes. Synthetic dopamine receptor-antagonists blocked both cellular responses with a similar rank order of potency (Feng et al., 1996; Reale et al., 1997a). In contrast, the rank order of potency of a range of synthetic receptor agonists that activated the different signaling pathways was not identical (Reale et al., 1997a).

DAMB/DopR99B mRNA was detected in heads as well as in legs and/or antennae by Northern blotting, suggesting that this receptor might be functional in both the central and peripheral nervous system (Han et al., 1996; Feng et al., 1996). In situ hybridization showed that DAMB transcripts are preferentially expressed in the perikarya of intrinsic mushroom body cells. Signals were almost absent in other parts of the brain, thoracic, and abdominal ganglia as well as in other tissues (Han et al., 1996). Staining of tissue sections with a polyclonal antibody also showed that the protein was preferentially expressed in the mushroom bodies. Strong labeling was found in the α- and β-lobes while the pedunculi and γ-lobes were less intensely stained. In con-

trast, immunoreactivity was absent in the calyces, which house the dendrites of intrinsic mushroom body neurons. The staining pattern of the DAMB-receptor is very similar to the expression pattern of a Ca^{2+}/calmodulin-regulated adenylyl cyclase encoded by the *rutabaga*-gene (Han et al., 1992). It has been suggested that this enzyme serves as a coincidence detector during conditioning in *Drosophila* and integrates cellular signals mediated by GPCR activation and/or the Ca^{2+}/calmodulin complex (Davis, 1993). Therefore, the co-localization of DAMB and the *rutabaga*-gene product in axons of mushroom body neurons as well as the ability of DAMB to activate adenylyl cyclase make this receptor an attractive candidate to mediate the effects of reinforcers during associative conditioning (Han et al., 1996).

Recently, a number of partial cDNA clones that probably code for biogenic amine receptors have been isolated from the honeybee by library scanning (Ebert et al., 1998). One of these fragments served to isolate a full-length cDNA clone (Am*BAR6*; Kokay et al., 1999; Humphries et al., unpublished data). The deduced amino acid sequence of Am*BAR6* (AmBAR6) shares ~70% identity with DAMB suggesting that it is the honeybee orthologue of the *Drosophila* dopamine receptor. Whole-mount in situ hybridization to worker honeybees and drones revealed that the expression pattern of Am*BAR6* is restricted to the mushroom bodies but differs between large and small diameter Kenyon (= intrinsic mushroom body) cells (Humphries and Ebert, 1998). In both sexes, Am*BAR6* mRNA is highly expressed in small-diameter Kenyon cells whereas expression levels in larger diameter Kenyon cells were variable and increased with the age of the worker bee (Humphries and Ebert, 1998). Whether AmBAR6 is a member of the D1-like dopamine receptor subfamily still awaits pharmacological characterization as well as identification of its intracellular transduction pathway.

Putative D2-Like Dopamine Receptors

Although dopamine D2-like receptors have not yet been cloned from *Drosophila* or other insects, pharmacological investigations suggest that they exist (Davis and Pitman, 1991; Granger et al., 1996; Yellman et al., 1997; Andretic and Hirsh, 2000). Receptors that display a D2-like pharmacology were identified in the honeybee. High affinity binding of [^{3}H]spiperone (K_D ~0.1 nM) was observed in brain homogenates (Kokay and Mercer, 1996). In addition to the pronounced pharmacological similarity with mammalian D2-like receptors, the honeybee [^{3}H]spiperone binding site also exhibits a phenolaminergic component (Kokay and Mercer, 1996). Therefore, it was assumed that the ligand will bind to other, most likely tyramine and/or octopamine receptors, as well. Incubation of brain sections with the radioligand showed that the binding sites are concentrated in the α- and β-lobes and calyces of the mushroom bodies (Kokay et al., 1998). In primary cultures of antennal lobe neurons, spiperone binding sites were also described (Kirchhof and Mercer, 1997; Kokay et al., 1999). A cDNA fragment (Am*BAR3*; Ebert et al., 1998) has recently been identified that is very similar to human dopamine D2-receptors and has led to the cloning of a full-length cDNA for a putative dopamine D2-like receptor from the honeybee (Kokay et al., 1999).

In summary, the dopamine receptors cloned from insects display almost unique pharmacological properties that set them apart from vertebrate receptors. The functional coupling of individual receptors to certain intracellular messenger systems is an alternative way to classify receptor subtypes. At present, two D1-like receptors have been characterized in *Drosophila* (DmDOP1, DAMB) and the honeybee (AmDOP1, AmBAR6). Whether additional subtypes are expressed should soon be answered with the availability of the complete genomic sequence of *Drosophila* (Adams et al., 2000). The presence of D2-like dopamine receptors in insects still has to be confirmed by additional cloning efforts and functional characterization of receptor candidates in both *Drosophila* and the honeybee.

RECEPTORS FOR TYRAMINE AND OCTOPAMINE

High concentrations of the phenolamines tyramine and octopamine are found in insect nervous tissue, whereas only trace amounts, if any, have been detected in vertebrate brains (for reviews see: Axelrod and Saavedra, 1977; David and Coulon, 1985). Little is known about the physiological role of tyramine in insects. It was assumed

that tyramine might only serve as biochemical precursor of octopamine rather than being a neuroactive substance itself. However, with the molecular cloning (see below) of specific tyramine receptors, and recent reports that attribute a role to tyramine in cocaine sensitization in *Drosophila* (McClung and Hirsh, 1999), a new picture is emerging.

In contrast to tyramine, the physiological role of octopamine has been thoroughly studied in a number of invertebrate species. Octopamine has been shown to act as a neurotransmitter, neuromodulator, and neurohormone, and modulates/regulates various behavioral patterns in insects (for reviews see: David and Coulon, 1985; Bicker and Menzel, 1989; Erber et al., 1993; Roeder, 1999). Often, octopamine is considered as a "fight or flight" hormone in insects and its physiological functions are compared with those of norepinephrine and epinephrine in vertebrates (Evans, 1993; Roeder, 1999).

In *Drosophila*, interneurons and efferent neurons constitute the octopaminergic neuronal population (Monastirioti et al., 1995; Monastirioti, 1999). In the larval CNS, all octopamine immunoreactive somata are localized in the midline of the ventral ganglion while in the adult CNS immunoreactivity is observed in clusters of both unpaired and bilateral neurons (Monastirioti et al., 1995; Monastirioti, 1999). When flies were fed with formamidines that probably bind to octopamine receptors they displayed impaired learning after classical conditioning (Dudai et al., 1987). Null mutations for the enzyme TβH, which is essential for the synthesis of octopamine (see Biogenic Amines Derived From Tyrosine), have been generated by P element insertion (Monastirioti et al., 1996). Interestingly, the animals are viable and do not show severe phenotypic alterations. Female flies, however, are sterile because they cannot deposit their eggs but sterility is rescued by feeding them with octopamine (Monastirioti et al., 1996). Although these results suggest that octopamine might not be as important for development and differentiation as dopamine (see Dopamine Receptors) it is currently unclear whether or not the loss of octopamine might have been functionally substituted by promiscuous binding of tyramine to octopamine receptors. The mutants that are unable to produce octopamine, however, will certainly help to unravel the contribution of octopamine in *Drosophila* learning and memory.

In the honeybee brain, five clusters of ~100 octopamine-immunoreactive somata were identified (Kreissl et al., 1994). Varicose octopaminergic fibers invade all parts of the brain and the suboesophageal ganglion except the pedunculi of the mushroom bodies and large parts of the α- and β-lobes (Kreissl et al., 1994). It has been shown that octopamine modulates many physiological functions such as the proboscis extension response (Mercer and Menzel, 1982; Braun and Bicker, 1992), sting response (Burrell and Smith, 1995), juvenile hormone release from the corpora allata (Rachinsky, 1994; Kaatz et al., 1994), and the discrimination of nestmates from unrelated bees (Robinson et al., 1999). Injection of octopamine into different areas of the CNS enhances neural activity and facilitates motor-reflexes (Erber et al., 1993; Erber and Kloppenburg, 1995; Kloppenburg and Erber, 1995; Pribbenow and Erber, 1996). One particular octopaminergic neuron, VUM_{mx1}, plays an important role in the reinforcement pathway during honeybee olfactory conditioning (Hammer, 1993). The VUM_{mx1} neuron depolarizes in response to the presentation of sucrose rewards to antennae and proboscis. Current injection into the VUM_{mx1} neuron or octopamine injection into either the antennal lobe or the calyces of the mushroom bodies can substitute for the sucrose reward during olfactory conditioning (Hammer and Menzel, 1998). It was concluded that octopamine is involved in selectively mediating the reinforcing but not the sensitizing or response-releasing function of the sucrose reward (Menzel et al., 1999).

Numerous pharmacological studies have been performed in locusts in order to classify octopamine receptor classes. According to their pharmacological properties and intracellular signaling pathways, four different classes were identified: OCT-1, OCT-2A, OCT-2B, and OCT-3 (for reviews see: Evans and Robb, 1993; Roeder, 1999). Activation of OCT-1 receptors induces an increase in $[Ca^{2+}]_i$, whereas activation of OCT-2A, OCT-2B, or OCT-3 receptors stimulates adenylyl cyclase and leads to increases in $[cAMP]_i$. In comparison to the locust, only a few studies were performed in *Drosophila* and the honeybee to determine the pharmacological properties of octopamine receptors (Table 3; Dudai and Zvi, 1984a; Degen et al., 2000). In *Drosophila* head homogenates, octopamine is a potent stimulator of

TABLE 3. Pharmacological properties of tyramine and octopamine receptors in *Drosophila melanogaster* and *Apis mellifera*

	Drosophila DmTYR in CHO-K1: [^{3}H]yohimbine (K_i[nM])	*Drosophila* DmTYR in COS-7: [^{3}H]yohimbine (K_i[nM])	*Drosophila* head homogenate: [^{3}H]yohimbine binding site (K_i[nM])	*Drosophila* head homogenate: [^{3}H]octopamine binding site (EC_{50}[nM])	*Apis* brain homogenate: [^{3}H]NC 5Z binding site (K_i[nM])
Biogenic amines					
Tyramine	1,400	1,200	380	300	51.4
DL-Synephrine	10,800	20,000	—	30	34.4
DL-Octopamine	129,200	40,000	62,100	6	13.4
Dopamine	137,900	50,000	—	5,000	—
(–)-Epinephrine	139,000	70,000	—	1,000	—
L(–)-Norepinephrine	—	150,000	—	700	—
Serotonin	75,000	175,000	—	20,000	—
Other ligands					
Yohimbine	6.2	5.5	4.6	4,000	—
Chlorpromazine	180	25	—	300	553
Phentolamine	2,200	85	350	20	48.7
Mianserin	1,200	100	—	—	0.73
Cyproheptadine	2,600	175	—	—	—
Metoclopramide	4,600	—	—	—	812
Clonidine	21,000	15,000	—	20	—

Values for DmTYR expressed in CHO-K1 cells are from Arakawa et al. (1990) and from Robb et al. (1994). Values for DmTYR expressed in COS-7 cells are from Saudou et al. (1990). Values for the *Drosophila* [^{3}H]yohimbine binding site are from Robb et al. (1994) and values for the *Drosophila* [^{3}H]octopamine binding site are from Dudai and Zvi (1984a). Values for the honeybee [^{3}H]NC-5Z binding site are from Degen et al. (2000). Note the higher affinity of tyramine compared to octopamine for the cloned DmTYR-receptor and the [^{3}H]yohimbine binding site in *Drosophila* head homogenates. This is in contrast to the nanomolar affinity of octopamine for native octopamine receptor binding sites.

adenylyl cyclase activity (Uzzan and Dudai, 1982). Interestingly, simultaneous application of both tyramine and octopamine reduces the effect of octopamine. This observation suggests that tyramine activates specific tyramine receptors that inhibit adenylyl cyclase and thereby reduce the stimulatory effect of octopamine (Uzzan and Dudai, 1982). In membrane homogenates of honeybee brains, octopamine also stimulates cAMP production (Blenau et al., 1996). In addition, injections of octopamine into the antennal lobe of the honeybee, evoke a rapid and transient activation of PKA (Hildebrandt and Müller, 1995). The effects of tyramine were also tested on membrane preparations from honeybee brain. When tyramine is applied at high concentrations, it activates adenylyl cyclase (EC_{50} of ~2.2 μM) but at low concentrations (0.1–1 μM) it attenuates forskolin-stimulated cAMP production (Blenau et al., 1996, 2000). Taken together, the results indicate that both octopamine and tyramine mediate their effects by binding to different members of the GPCR family.

The distribution of binding sites for [^{3}H]octopamine in tissue sections of honeybee brain has been analyzed with autoradiographic methods (for a review see Erber et al., 1993). Specific and high labeling densities were detected in the mushroom bodies, especially in the pedunculus and in the α- and β-lobes. Interestingly, these brain regions are not innervated by octopaminergic neurons (Erber et al., 1993; Kreissl et al., 1994). Phentolamine displaced ~93% of [^{3}H]octopamine binding in all brain areas except the mushroom bodies (~70% displacement). These results suggested that octopamine receptors in the mushroom bodies may be pharmacologically different from those in the rest of the brain (Erber et al., 1993).

Drosophila DmTYR- and the Honeybee AmTYR1-Receptor

A gene encoding the first member of the tyramine/octopamine receptor family from *Drosophila* (Dm*oct/tyr*) was independently cloned by two groups (Arakawa et al., 1990; Saudou et al., 1990). The deduced amino acid sequence of DmOCT/TYR is highly homologous to mammalian α_2-adrenergic receptors (Arakawa et al., 1990). The functional coupling to intracellular sig-

naling pathways was examined after heterologous expression of the gene in either Chinese hamster ovary (CHO-K1; Arakawa et al., 1990) or COS-7 cells (Saudou et al., 1990). Application of tyramine or octopamine attenuated forskolin-stimulated adenylyl cyclase activity (Arakawa et al. 1990; Saudou et al., 1990). The affinity of tyramine to the receptor is ~12 times higher than that of octopamine (Table 3; Saudou et al., 1990; Robb et al., 1994). This led to the suggestion that the gene most likely encodes a functional tyramine receptor (Saudou et al., 1990). In subsequent biochemical studies, it was shown that the receptor mediates both inhibition of adenylyl cyclase activity and elevation of $[Ca^{2+}]_i$ (Robb et al., 1994). Tyramine is about two orders of magnitude more potent than octopamine in inhibiting forskolin-induced cAMP accumulation, whereas octopamine is slightly more potent in elevating $[Ca^{2+}]_i$ (Robb et al., 1994). Interestingly, when expressed in *Xenopus* oocytes, stimulation of DmOCT/TYR with either ligand led to an increase of $[Ca^{2+}]_i$ but failed to inhibit adenylyl cyclase activity (Reale et al., 1997b). Thus, this *Drosophila* receptor displays "agonist-specific coupling to different second messenger systems," which seems to depend on the specific supply of G proteins provided by the different cell types used for heterologous expression (Robb et al., 1994; Reale et al., 1997b).

Recently, a *Drosophila* mutant (*hono*) was identified that shows impaired olfactory avoidance behavior to repellents (Kutsukake et al., 2000). This strain is a β-Gal enhancer-trap line generated using a *P*-element mutagenesis approach aimed at identifying novel olfactory mutants. Standard cloning techniques revealed that the *P*-element is located in the promoter region of the Dm*oct/tyr*-gene and causes reduced expression of the Dm*oct/tyr* mRNA (Kutsukake et al., 2000). In addition to the reduced olfactory sensitivity, the mutation also reduced the electrical responses to tyramine at the neuromuscular junction in the larval body-wall whereas responses to octopamine remained normal (Kutsukake et al., 2000). Since *hono* is the first biogenic amine receptor mutant identified in invertebrates and the effects caused by the *hono* mutant are specifically correlated with impaired functions of tyramine, the Dm*oct/tyr*-gene should be considered as a "true" tyramine receptor gene (Dm*tyr*).

The expression pattern of the *hono*-gene was examined by staining for β-Gal positive cells. In addition to the antennae, many areas of the adult brain and thoracic ganglion were stained (Kutsukake et al., 2000). This result agrees well with the mRNA expression pattern that was independently determined by in situ hybridization to tissue sections of wild-type flies (Hannan and Hall, 1996). In third instar larvae of the *hono* mutant, β-Gal staining was detected in the olfactory organ (dorsal organ), in the CNS, and along the morphogenetic furrow of the eye-antennal disc. The results strongly suggest that the DmTYR-receptor is important in various aspects of olfactory information processing (Kutsukake et al., 2000).

An orthologue of the *Drosophila* tyramine receptor gene was cloned from *Apis mellifera* (Am*tyr1*; Blenau et al., 2000). Pronounced sequence conservation of 83.2 and 78.2% was observed between the deduced amino acid sequence of Am*tyr1* and a tyramine receptor cloned from *Locusta migratoria* (LocTYR; Vanden Broeck et al., 1995) and an octopamine receptor cloned from *Heliothis virescens* (K50Hel = HelOCT; von Nickisch-Rosenegk et al., 1996), respectively. Multiple alignments of invertebrate receptor sequences showed that AmTYR1, DmTYR, LocTYR, and HelOCT form a distinct group within the biogenic amine receptor family (see Fig. 8). When stably expressed in HEK 293 cells, the AmTYR1-receptor attenuated forskolin-induced cAMP production after stimulation with tyramine in a dose-dependent manner. The EC_{50} for tyramine was ~130 nM. Octopamine also reduced cAMP production in the transfected cell line but was both less potent (EC_{50} ~3 μM) and less efficacious than tyramine (Blenau et al., 2000). Similar to the *Drosophila* Dm*tyr*-gene (Hannan and Hall, 1996; Kutsukake et al., 2000), Am*tyr1* mRNA is abundantly expressed in many neurons of the honeybee brain, including neurons of the optic lobes, mushroom body intrinsic neurons, and neurons of the deutocerebrum. Whether the honeybee AmTYR1-receptor participates in olfactory signaling as has been demonstrated for the *Drosophila* DmTYR-receptor is currently unknown.

Drosophila OAMB- and the Honeybee AmOCT1- (= AmBAR1-) Receptor

A cDNA encoding an octopamine receptor (OAMB) was isolated from a *Drosophila* head specific library (Han et al., 1998). The deduced amino

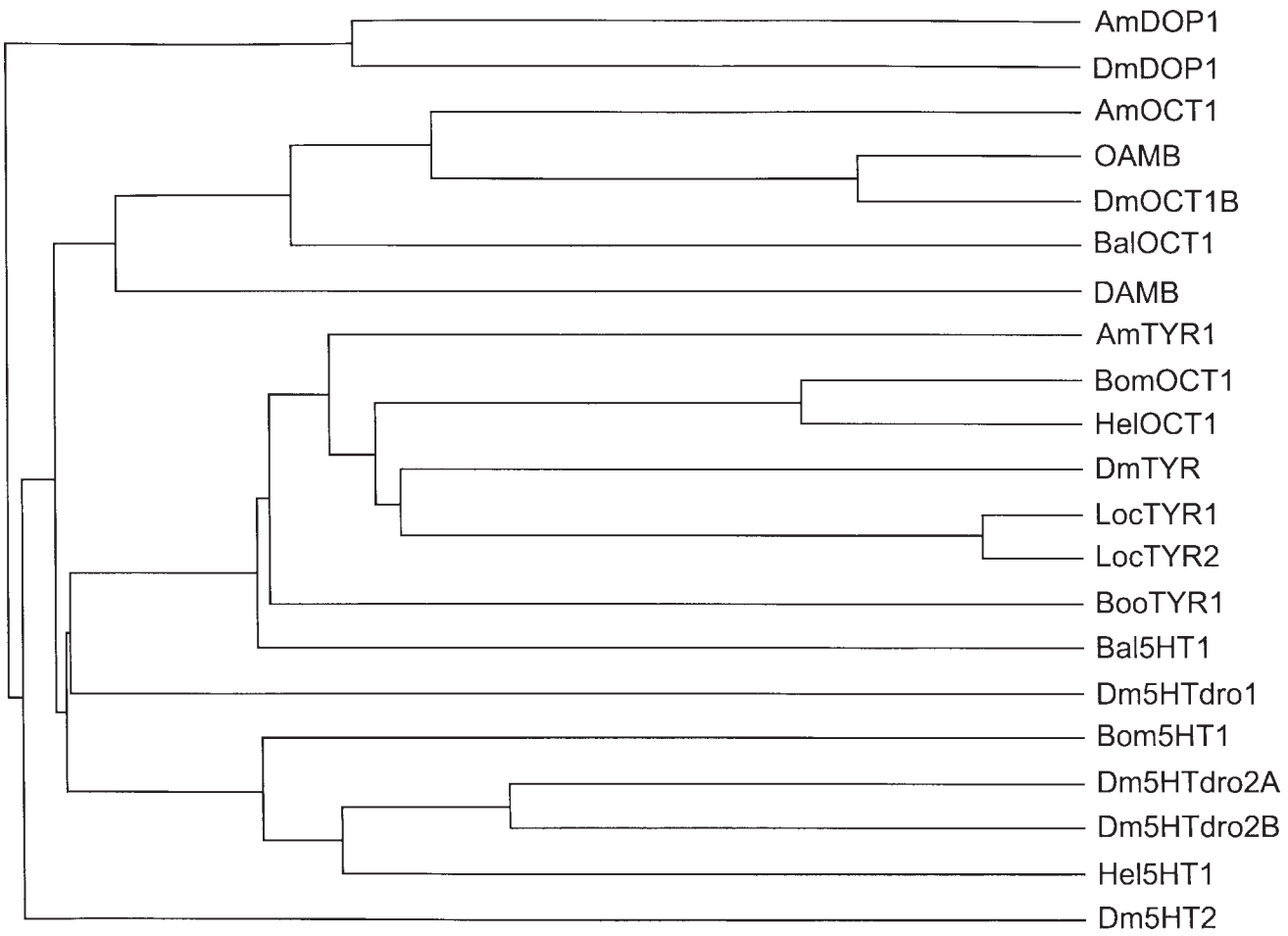

Fig. 8. Dendrogram of arthropod biogenic amine receptors. Sequence alignment was done with the CLUSTAL program of PCGENE software (version 6.6; IntelliGenetic), using the complete amino acid sequence of each receptor. The receptor sequences followed by their GenBank/EMBL accession numbers (#) are listed in the order illustrated: *Apis mellifera* dopamine D1 (AmDOP1, #Y13429), *Drosophila melanogaster* dopamine D1 (DmDOP1, #X77234), *A. mellifera* octopamine (AmOCT1), *D. melanogaster* octopamine (OAMB, #AF065443; DmOCT1B, #AJ007617), *Balanus amphitrite* (barnacle) putative octopamine (BalOCT1, #D78363), *D. melanogaster* dopamine (DAMB, #U61264), *A. mellifera* tyramine (AmTYR1, #AJ245824), *Bombyx mori* octopamine (BomOCT1, #Q17232), *Heliothis virescens* octopamine (HelOCT1, Q25188), *D. melanogaster* tyramine (DmTYR, #M60789), *Locusta migratoria* tyramine (LocTYR1, #Q25321; LocTYR2, #Q25322), *Boophilus microplus* (cattle tick) putative tyramine (BooTYR1, #AJ010743), *B. amphitrite* putative serotonin (Bal5HT1, #D83547), *D. melanogaster* serotonin (Dm5HTdro1, #P20905), *B. mori* putative serotonin (Bom5HT1, #Q25414), *D. melanogaster* serotonin (Dm5HTdro2A, #Z11489; Dm5HTdro2B, #Z11490), *H. virescens* putative serotonin (Hel5HT1, X95605), and *D. melanogaster* serotonin (Dm5HT2, X81835).

acid sequence is very similar to those of mammalian adrenergic as well as invertebrate tyramine and dopamine receptors. One unique feature of OAMB is the extension of EL2. This loop usually contains ~20 amino acids but in OAMB consists of 130 residues. The functional significance of this extension is still unknown (Han et al., 1998). Expression of the OAMB-gene in either *Drosophila* S2 or HEK 293 cells led to an increase in $[cAMP]_i$. The EC_{50} for octopamine was $\sim 1.9 \times 10^{-7}$ M (Han et al., 1998). Tyramine was much less effective, strongly suggesting that the gene encodes a functional octopamine receptor. In addition to cAMP production, octopamine also induced an increase in $[Ca^{2+}]_i$ in OAMB expressing cells although only a relatively high concentration of octopamine (10^{-5} M) was tested (Han et al., 1998). Since octopamine receptors were originally classified according to their coupling to either $[cAMP]_i$ or to $[Ca^{2+}]_i$ (see above), the OAMB-receptor is difficult to ascribe to any one of the existing receptor classes (Han et al., 1998).

The tissue distribution of the OAMB mRNA and protein were examined by in situ hybridization and immunohistochemistry (Han et al., 1998). OAMB transcripts were detected in perikarya of intrinsic mushroom body neurons, and in two clusters of cells in the anterior brain cortex in proximity to the mushroom body lobes. In addition, cells scattered throughout the central brain and medulla were stained. Immunoreactivity was observed in the calyces, pedunculi, α-, β-, and γ-lobes of the mushroom bodies, and in the ellipsoid body of the central complex (Han et al., 1998). Since OAMB can activate adenylyl cyclase and is preferentially expressed in the mushroom bodies, it was suggested that this receptor could participate in olfactory learning in *Drosophila* (Han et al., 1998).

In the honeybee, the octopaminergic VUM_{mx1} neuron is involved in olfactory conditioning (Hammer, 1993) and it is assumed that specific octopamine receptors modulate the biochemical signaling cascades within the olfactory pathway. To date, sequence information on only one octopamine receptor gene has emerged (Am*BAR1*; Ebert et al., 1998; Am*oct1*, Grohmann et al., 2000). The deduced amino acid sequence displays high similarity to the OAMB-gene from *Drosophila*. When expressed in HEK 293 cells, AmOCT1 activation leads to an increase in $[Ca^{2+}]_i$ and $[cAMP]_i$ (Grohmann et al., 2000). In contrast to OAMB, submicromolar concentrations of octopamine are sufficient to induce the Ca^{2+} response whereas micromolar or higher concentrations are necessary to activate adenylyl cyclase. In situ hybridization to honeybee brain sections showed that the gene is expressed in some perikarya of intrinsic mushroom body neurons, in somata of the antennal and optic lobes, and in somata of the suboesophageal ganglion (Grohmann et al., 2000).

Tyramine and octopamine receptor genes have also been cloned from *Locusta migratoria* (GenBank accession nos. Q25322, Q25321; Vanden Broeck et al., 1995) as well as *Bombyx mori* and *Heliothis virescens* (Q17323 and Q25188; von Nickisch-Rosenegk et al., 1996). The physiological role of these receptors still awaits identification.

SEROTONIN RECEPTORS

Serotonin (see Serotonin Is Derived From Tryptophan) serves as a neurotransmitter or neuromodulator in most animal species. Serotonin regulates or modulates a wide variety of behaviors such as aggression in lobsters, feeding and learning in snails, locomotion in lampreys, as well as sleep, appetite, and mood in mammals (for reviews see Bicker and Menzel, 1989; Hen, 1992; Boess and Martin, 1994).

In *Drosophila*, as in most arthropods, the majority of serotonergic neurons are interneurons in the brain and in the ventral nerve cord (Vallés and White, 1988). The total number of these neurons is small (~100 cells in *Drosophila*) but they send projections to most parts of the nervous system. Serotonergic innervation is also found in the pharyngeal muscles as well as in the ring gland, the endocrine organ of the larvae (Vallés and White, 1988), suggesting that serotonin modulates feeding behavior and neuroendocrine activity of *Drosophila*. During *Drosophila* development, a peak in serotonin concentration precisely coincides with the onset of germband extension in gastrulating embryos. The peak of serotonin synthesis strictly depends on the maternal deposition of biopterins into the egg and the zygotic synthesis of TRH and DDC (see Serotonin Is Derived From Tryptophan; Colas et al., 1999a). Mutants with impaired serotonin synthesis are embryonic lethal and/or display abnormal gastrulation movements and cuticular defects (Colas et al., 1999a).

The distribution of serotonin-immunoreactivity has been intensively studied in the brain of adult (Schürmann and Klemm, 1984; Nässel et al., 1985; Schäfer and Bicker, 1986; Rehder et al., 1987) and pupal worker honeybees (Seidel and Bicker, 1996). A small set of ~75 cell bodies gives rise to neuronal branches that arborize into most parts of the brain neuropil. The adult set of serotonergic neurons consists of both persistent embryonic neurons and neurons that differentiate during pupal metamorphosis. Behavioral experiments have shown that serotonin often functionally antagonizes the effects of octopamine and reduces or blocks rhythmic behavior and reflexes (Erber et al., 1993; Erber and Kloppenburg, 1995; Kloppenburg and Erber, 1995; Pribbenow and Erber, 1996; Blenau and Erber, 1998). Serotonin also has profound effects on learning and memory in impairing acquisition and retrieval of learned behaviors (for reviews see Bicker and Menzel, 1989; Menzel et al., 1994).

In vertebrates, serotonin binds to and activates different types of membrane receptors. From 14 receptor genes cloned, one forms an ion channel (5-HT_3). All other genes encode GPCRs. Five receptors inhibit cAMP production (5-$HT_{1A/B/D/E/F}$), three receptors induce cAMP production (5-$HT_{4/6/7}$), three receptors lead to an increase in $[Ca^{2+}]_i$ (5-$HT_{2A/B/C}$), and for two receptors (5-$HT_{5A/B}$) the intracellular signaling pathways have not yet been determined (Hoyer and Martin, 1997). Besides their functional coupling characteristics, serotonin receptors were classified according to their binding properties to specific (synthetic) compounds (for a review see: Boess and Martin, 1994).

Serotonin-sensitive binding sites have also been characterized in *Drosophila* head homogenates (Table 4; Dudai and Zvi, 1982, 1984b) and in membrane preparations of honeybee brains (Table 4; Blenau et al., 1995b). Autoradiographic studies in the honeybee showed that receptor density was low in the antennal lobes but high in the calyces of the mushroom bodies (for a review see: Erber et al., 1993). Similar to the different signaling pathways described for vertebrate serotonin receptors, it was shown in membrane homogenates of honeybee brains that serotonin not only stimulates cAMP production but also inhibits forskolin-induced cAMP production (Blenau et al., 1996). Molecular cloning and functional characterization of serotonin receptors from *Drosophila* revealed that serotonin also operates through different signaling pathways in the fly (Hen, 1992).

Drosophila Dm5HTdro1-Receptor

Using a homology screening approach, the first *Drosophila* serotonin receptor gene (Dm5HTdro1; Witz et al., 1990) was cloned from a head specific cDNA library. The deduced amino acid sequence shares considerable sequence similarity with mammalian 5-HT_7-receptors (Gerhardt and van Heerikhuizen, 1997). A hydropathy profile analysis revealed an interesting feature in the N-terminus of Dm5HTdro1. In addition to the seven TMs, an eighth hydrophobic stretch was identified (Witz et al., 1990). This segment is theoretically long enough to span the membrane as a TM but could also serve as an internal signal sequence that is cleaved off during maturation of the protein. However, an additional TM segment would perturb the transmembrane architecture of the receptor and cause exposure of usually intracellular loops to the external surface. Such an orientation would render the receptor non-functional. This, however, is not the case as has been demonstrated using heterologous expression of the Dm5HTdro1-gene in either mouse fibroblasts (NIH 3T3 cells; Witz et al., 1990) or insect Sf9 cells (Obosi et al., 1996). In both cell lines, application of serotonin or receptor agonists led to an increase in $[cAMP]_i$. Stimulation of adenylyl cyclase is a common feature of vertebrate 5-$HT_{4/6/7}$-receptors. Therefore, Dm5HTdro1 not only shares sequence similarity to this group of vertebrate serotonin receptors, but also activates the same intracellular signaling pathway. However, as has already been shown for other invertebrate receptors, the pharmacological properties of Dm5HTdro1 differ quite significantly from those of vertebrate 5-$HT_{4/6/7}$-receptors (Table 4; Saudou et al., 1992). Particularly striking is the low affinity of 8-OH-DPAT for the *Drosophila* receptor (Table 4).

Another sequence motif in the N-terminus of Dm5HTdro1 provides a clue to a possible physiological role for this receptor in the fly. A stretch of Ser-Gly repeats is located between the newly identified eighth hydrophobic domain and the first putative TM. Repetitive stretches of Ser-Gly or Thr-Gly motives have been implicated as attachment sites for posttranslational modifications with glycosaminoglycans. Similar motives were already identified in biological clock genes like *period* in *Drosophila* or *frequency* in *Neurospora*. It was assumed, therefore, that Dm5HTdro1 might also be linked to glycosaminoglycans (Witz et al., 1990). A function of this modification could be to localize the receptor in a specialized compartment or subdomain of the cell.

Drosophila Dm5HTdro2A- and Dm5HTdro2B-Receptors

Molecular cloning of two additional, closely related serotonin receptor genes again was achieved by an homology based screening approach (Saudou et al., 1992). The deduced amino acid sequences of Dm5HTdro2A- and Dm5HTdro2B-receptors share 84.3% sequence homology. Both genes encode GPCRs that also share sequence similarity with mammalian 5-HT_{1A}-receptors (Gerhardt and van Heerikhuizen, 1997). When the *Drosophila*

TABLE 4. Pharmacological Properties of Serotonin Receptors in *Drosophila melanogaster* and *Apis mellifera*

	Drosophila Dm5HTdro1 in COS-7: [^{125}I]LSD binding (K_i[nM])	*Drosophila* Dm5HTdro2A in COS-7: [^{125}I]LSD binding (K_i[nM])	*Drosophila* Dm5HTdro2B in COS-7: [^{125}I]LSD binding (K_i[nM])	*Drosophila* Dm5HT2 in COS-1: [^{125}I]DOI binding (K_i[nM])	*Drosophila* head homogenate: [^{3}H]serotonin binding site (IC_{50}[nM])	*Apis* brain homogenate: serotonin-sensitive [^{3}H]LSD binding site (K_i[nM])
Biogenic amines						
Serotonin	1,600	16,000	2,100	15	1.4 130	2.6
Tryptamine	—	—	—	1,580	700	130
5-Methoxytryptamine	—	—	—	5,010	700	43
Tyramine	>200,000	>200,000	>200,000	≥100,000	80,000	>>10,000
DL-Octopamine	>200,000	>200,000	>200,000	≥100,000	—	>>10,000
Dopamine	>200,000	>200,000	>200,000	≥100,000	200,000	47,000
L(–)-Norepinephrine	>200,000	>200,000	>200,000	—	—	>>10,000
Histamine	>200,000	>200,000	>200,000	≥100,000	—	>>10,000
Other ligands						
LSD	0.44	0.26	0.31	—	—	0.89
Dihydroergocryptine	13	11	3.8	—	—	—
S(+)-Butaclamol	32	330	64	—	—	>10,000
R(–)-Butaclamol	31,000	14,000	30,000	—	—	>>10,000
Prazosin	9,800	250	180	—	—	—
Methysergide	1,200	1,400	720	79	40	22
Yohimbine	32,000	18,000	9,800	398	50,000	—
Mianserin	—	—	—	1,995	—	1,500
8-OH-DPAT	106,000	43,000	27,000	12,600	—	2,300

Values for Dm5HTdro1, Dm5HTdro2A, and Dm5HTdro2B are from Saudou et al. (1992). Values for Dm5HT2 are from Colas et al. (1995). Values for the *Drosophila* [^{3}H]serotonin binding site are from Dudai and Zvi (1984b) and values for the serotonin-sensitive [^{3}H]LSD binding site of the honeybee are from Blenau et al. (1995b). Note the low affinity of 8-OH-DPAT for the *Drosophila* Dm5HTdro1-receptor.

genes were mapped on the chromosomes, the loci were located within the same chromosomal band. This suggested that the genes most likely arose by duplication of a common ancestor gene. In addition to their sequence homology to vertebrate 5-HT_1-receptors, both *Drosophila* receptors also share the intracellular transduction pathway with their vertebrate counterparts. Application of serotonin to heterologously expressed receptors decreased forskolin-stimulated cAMP production with an EC_{50} of ~3×10^{-8} M and ~1.8×10^{-8} M for Dm5HTdro2A and Dm5HTdro2B, respectively. In addition to the inhibition of adenylyl cyclase, both receptors moderately activate PLC in response to serotonin (Saudou et al., 1992). The specificity of these receptors for serotonin was confirmed by direct radioligand binding assays (Table 4; Saudou et al., 1992). Serotonin efficiently displaced the radioligand [^{125}I]LSD from both receptors whereas dopamine, octopamine, tyramine, and histamine were ≥100-fold less efficient. The expression patterns of the genes were examined by Northern blotting and in situ hybridization (Saudou et al., 1992). Transcripts were detected in RNA preparations from embryos, larvae, and adult heads. Expression in the embryo was restricted to the CNS. The two receptors, however, were expressed in distinct cell populations. While Dm5HTdro2A is predominantly found in VUM neurons, cells that are involved in axonal guidance during commissure formation in embryonic development (Klämbt et al., 1991), the Dm5HTdro2B most likely is expressed in motor neurons. Although the subcellular distribution of the receptor proteins has not yet been examined, it was suggested that both receptors could control motor activity in the fly (Saudou et al., 1992).

Drosophila Dm5HT2-Receptor

Isolation of the Dm5HT2-receptor gene again was achieved by a homology screening approach (Colas et al., 1995). When compared to vertebrate serotonin receptors, the Dm5HT2 amino acid sequence displayed striking similarity to 5-HT_2-receptors (Gerhardt and van Heerikhuizen, 1997). Activation of these receptors causes an increase in $[Ca^{2+}]_i$. The transduction mechanism of Dm5HT2 has not yet been reported. In comparison to the deduced amino acid sequences of mammalian 5-HT_2-receptors, the only difference in Dm5HT2 is the longer size of the N-terminus. A partial deletion of the N-terminus of Dm5HT2 does not alter its pharmacological properties but significantly increases its expression pattern in mammalian cell lines (Colas et al., 1997). Therefore, it is likely that the long N-terminus interferes with proper processing/maturation of the receptor in heterologous expression systems. The pharmacological properties of the wild-type Dm5HT2-receptor were determined after expression of the gene in COS-1 cells (Table 4; Colas et al., 1995, 1997). Binding studies showed that the pharmacological profile of the receptor, in addition to its striking sequence similarity, correlated well with that of mammalian 5-HT_2-receptors, but not with any other cloned serotonin receptor (Colas et al., 1995, 1997).

The expression pattern of the Dm5HT2-gene was examined by in situ hybridization and quantitative RT-PCR (Colas et al., 1995). The gene is expressed during embryogenesis as well as in the larval and adult CNS. Interestingly, the mRNA is already found after 3 h of embryonic development. In the embryo, the mRNA is present in seven evenly spaced transverse stripes along the antero-posterior axis. This pattern is very similar to that of pair-rule genes. Comparison with expression patterns of different pair-rule genes finally showed that Dm5HT2 is co-expressed with *fushi-tarazu* in the even-numbered parasegments (Colas et al., 1995). It is most likely that the Dm5HT2-receptor is necessary for proper germband extension. Mutants that do not express Dm5HT2 are embryonic lethal. They show a significant delay in germband extension, which results in uncoupling of ectodermal elongation from endoderm and mesoderm invaginations (Colas et al., 1999b).

A serotonin receptor gene was also cloned from *Bombyx mori* (Q17239; von Nickisch-Rosenegk et al., 1996). All cloned insect serotonin receptors are members of the GPCR family. A serotonin-gated ion channel comparable to the mammalian 5-HT_3-receptor has not been described in insects, so far. While amino acid sequences and transduction mechanisms are often well conserved between insect and mammalian receptors, the pharmacological properties often differ considerably and do not allow insect receptors to be classified according to existing mammalian schemes (Saudou et al., 1992; Blenau et al., 1995b).

HISTAMINE RECEPTORS

Histamine has been established as the major neurotransmitter that is released from insect photoreceptor cells in response to illumination (for reviews see: Nässel, 1991, 1999). In *Drosophila,* histamine-like immunoreactivity was also detected in a small number (18–24) of neurons within the brain (Nässel et al., 1990; Sarthy, 1991; Pollak and Hofbauer, 1991; Nässel and Elekes, 1992). Many of these neurons have extensive bilateral arborizations that innervate distinct regions of neuropil. It has also been shown in *Drosophila* that almost all mechanosensory neurons of imaginal hair sensilla contain histamine (Buchner et al, 1993). The brain of the honeybee contains about 150 histaminergic neurons (Bornhauser and Meyer, 1997). The axons of these neurons innervate most parts of the protocerebrum except the mushroom bodies (Bornhauser and Meyer, 1997). Photoreceptor fibers terminating either in the lamina or in the medulla as well as axons emanating from ocellar photoreceptors also contain histamine (Bornhauser and Meyer, 1997).

Histamine synthesis has been experimentally impaired by mutations in the gene encoding HDC (see Histamine Is Derived From Histidine; Burg et al., 1993). Although the mutants (*hdc*) are devoid of histamine, they are viable. This suggests that histamine does not have a vital function during development or in the adult. However, HDC null mutants are blind (Burg et al., 1993). This is most likely due to non-existing synaptic transmission between photoreceptor terminals and postsynaptic monopolar cells in the lamina or medulla. In addition to its role in the visual system, analysis of several other *hdc* alleles suggested that histamine is important for mechanosensory transduction as well (Melzig et al., 1996). It was shown recently that a histamine-selective uptake mechanism can restore the wild-type functions of photo- and mechanoreceptors in *hdc* mutants (Melzig et al., 1998). Besides release from receptor-neurons, histamine is also important for communication between interneurons. In the cricket *Gryllus bimaculatus*, histamine has also been shown to inhibit an auditory interneuron within the prothoracic ganglion (ON1; Skiebe et al., 1990). Therefore, histamine should be considered as a sensory transmitter as well as a modulator of interneurons in insects.

So far, three histamine receptor genes have been cloned from vertebrates. They all belong to the family of GPCRs. Histamine H_1-receptors lead to an increase in $[Ca^{2+}]_i$, whereas histamine H_2-receptors activate and histamine H_3-receptors inhibit adenylyl cyclase (Hill et al., 1997). Selective agonists and antagonists are available that allow these different receptor subtypes to be differentiated pharmacologically.

No invertebrate histamine receptor homologues have yet been cloned. However, binding studies with different radioligands, as well as attempts to purify receptors from membrane preparations, have already been performed in the locust (Elias et al., 1984; Roeder, 1990; Roeder et al., 1993, 1995). At present, it is not clear whether invertebrate histamine receptors belong to the family of GPCRs. Since electrophysiological investigations have shown that histamine released from photoreceptors activates chloride currents in postsynaptic monopolar cells (Hardie, 1987, 1989; Skingsley et al, 1995), one could speculate that invertebrates possess (only) histamine-gated ion channels. Nevertheless, ongoing and already completed genome projects will certainly assist us to answer this question in the near future.

CONCLUSIONS

Biogenic amines are important mediators and/or regulators of neuronal signaling in the central and peripheral nervous system of insects. A prerequisite to understanding the physiological role of biogenic amine signaling systems is the molecular characterization of biogenic amine receptors and their downstream reaction partners. The application of molecular biological methods has greatly enhanced our knowledge of the receptor polypeptides. As in vertebrates, multiple receptor subtypes mediate the actions of biogenic amines in insects. All biogenic amine receptors identified so far in invertebrates belong to the superfamily of GPCRs and activation of heterologously expressed receptor polypeptides generally activate the same intracellular signaling pathways as native receptors do in vivo. However, several receptor subtypes still appear to be “missing” as not all of the pharmacological and signaling properties of biogenic amine receptors described in vivo are covered by the receptors cloned so far.

However, the availability of complete genomic sequences (*Drosophila melanogaster*; Adams et al., 2000) and ongoing molecular research (*Apis mellifera*; Maleszka, 2000) will allow such gaps to be filled in the near future. Combining the experimental advantages of both insect species, i.e., honeybees to study social behavior and the neuronal and biochemical basis of learning and memory (Menzel and Müller, 1996, Meller and Davis, 1996, Hammer, 1997), and *Drosophila* with its genetic potential, will certainly improve our understanding of the behavioral, developmental, and physiological role of individual biogenic amine-regulated transduction pathways.

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Biogenic Amines

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Biogenic amines are important messenger substances and regulators of cell functions. In insects, these small organic compounds act as neurotransmitters, neuromodulators, and neurohormones. Biogenic amines control endocrine and exocrine secretion, the contraction properties of muscles, the activity of neurons, and the generation of motor patterns. In addition, certain biogenic amines are involved in learning and the formation of memory. Biogenic amines mediate these diverse cellular and physiological effects by binding to specific membrane proteins that primarily belong to the superfamily of G-protein-coupled receptors.

Specialized Terms

Ca^{2+} signaling Change in the intracellular Ca^{2+} concentration, through the release of Ca^{2+} ions from intracellular stores by the opening of ligand-gated ion channels or the entry of Ca^{2+} ions into the cell through different types of Ca^{2+}-selective channels located in the plasma membrane, that plays a role in the regulation of various cellular processes, including cell metabolism, gene expression, cytoskeletal dynamics, and neurotransmission.

cyclic AMP Cyclic derivative of adenosine monophosphate that is synthesized from ATP by adenylyl cyclase. Intracellular second messenger involved in the regulation or modulation of ion channels, protein kinase activity, and gene expression.

G-protein-coupled receptors Integral membrane proteins that constitute a large family of neurotransmitter, hormone, or olfactory receptors. Characterized by seven transmembrane regions. When agonists bind to these receptors trimeric GTP-binding (G) proteins are activated that then regulate the activity of intracellular secondary effectors, which change intracellular concentrations of second messengers or ion channel activity.

neurohormone Small organic or peptidergic substance that is produced in neurosecretory cells. Released into the hemolymph at special regions called neurohemal organs. Transported to target tissues with the hemolymph.

neuromodulator Neuroactive substance that is released by synaptic terminals. Simultaneously acts on large numbers of cells in the proximity of the releasing cell and modifies the properties of synaptic transmission and the properties of target cells.

neurotransmitter Chemical substance that is released from the presynaptic endings of a neuron. Transmits information across the synaptic cleft to specific receptors located on the surface of postsynaptic cells.

phosphorylation Transient, reversible posttranslational modification of proteins in which the terminal phosphate group of ATP is transferred to specific residues of a polypeptide by kinases and often alters the properties of the protein.

second messenger Intracellular substance, such as Ca^{2+}, cyclic AMP, inositol-1,4,5-trisphosphate, that modifies or modulates cellular responses. Concentration changes in response to activation of G-protein-coupled receptors.

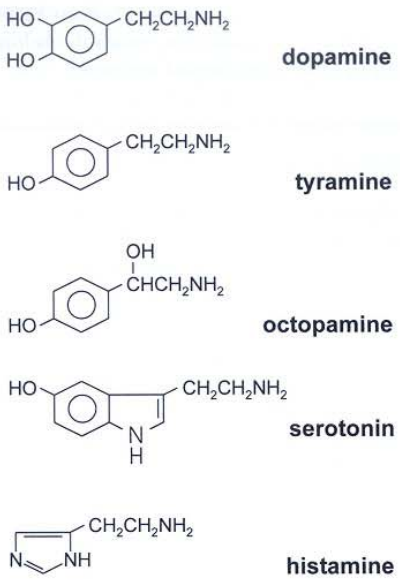

FIGURE 1 Biogenic amines of invertebrates. In insects five substances have been identified as biogenic amines: dopamine, tyramine, octopamine, serotonin, and histamine.

BIOSYNTHESIS OF BIOGENIC AMINES

Biogenic amines are involved in a variety of regulatory functions. Five primary amines are considered biogenic amines in invertebrates: histamine (HA), serotonin (5-HT), dopamine (DA), tyramine (TA), and octopamine (OA) (see Fig. 1). These small organic compounds are synthesized from three different amino acids by single to multistep enzymatic reactions.

LOCALIZATION OF BIOGENIC AMINES

Aminergic systems in insects and vertebrates are quite different. In insects, OA and TA are present in relatively high concentrations, whereas they appear to have only minor significance in vertebrates. In contrast, the catecholamines norepinephrine and epinephrine are important chemical messengers in vertebrates, whereas in the insect nervous system they are detected only in very low concentrations if at all. Several additional catecholamines are involved in the process of cuticle tanning, hardening, and sclerotization in insects. These catecholamines are cross-linking reagents for cuticle proteins and chitin.

Considerable physiological, biochemical, and histochemical evidence suggests that HA, 5-HT, DA, OA, and TA act as transmitters or modulators in the central and peripheral nervous systems of insects. Antisera to HA, 5-HT, DA, and OA often label interneurons that have wide branching patterns within the central nervous system, sometimes innervating neuropils bilaterally. The dorsal and ventral unpaired median neurons, which can contain OA, are well-known examples of such large-field cells. Amine-containing neurons with large arborizations are well suited to act on large groups of other neurons simultaneously. In addition to these large-field cells there are small-field aminergic neurons, especially in the central complex and in the optic lobes. The neuroanatomy of these cells suggests that they communicate with a limited number of target cells.

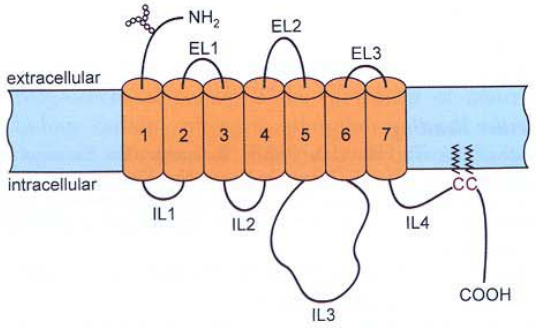

FIGURE 2 Transmembrane topography of G-protein-coupled receptors. The polypeptide spans the membrane seven times. The transmembrane regions (TM 1–7) are depicted as cylinders. The N-terminus (NH_2) is located extracellularly and often contains glycosylated residues (o). The C-terminus (COOH) is located intracellularly. The membrane-spanning regions are linked by three extracellular loops (EL1–EL3) that alternate with three intracellular loops (IL1–IL3). Posttranslational palmitoylation of cysteine residues (C) in the cytoplasmic tail creates a fourth intracellular loop (IL4).

BIOGENIC AMINE RECEPTORS

Biogenic amines bind to specific integral membrane receptors belonging predominantly to the superfamily of G-protein-coupled receptors. Physicochemical, biochemical, and immunochemical investigations show that these polypeptides share the common motif of seven transmembrane (TM) segments (Fig. 2). The N-terminus is located extracellularly, whereas the C-terminus is located intracellularly. The N-terminus is the target of a common posttranslational modification. In this part of the polypeptide consensus sequence motifs are often glycosylated. The membrane-spanning regions are linked by three extracellular loops (EL) that alternate with three intracellular loops (IL). Cysteine residues in the C-terminus of the polypeptides are the target of posttranslational palmitoylation. This modification creates a fourth intracellular loop.

A receptor is activated after binding of the specific biogenic amine in a binding pocket formed by the TM regions in the plane of the membrane. Individual residues in TM3, TM5, and TM6 were shown to participate in ligand binding. Once the ligand is bound, the receptor changes its conformation. This structural alteration usually is registered by intracellular trimeric GTP-binding proteins (G proteins). Residues that reside in close proximity to the plasma membrane in IL2, IL3, and IL4 of the receptor proteins determine the specificity and efficacy of the interaction between receptor and G protein.

GENERAL FUNCTIONS OF BIOGENIC AMINES

Biogenic amines have diverse functions controlling all phases of the life cycle of an insect. They are important chemical messengers during embryonic and larval development and they participate in the synaptic organization of the brain in the adult. As neuroactive substances they act on sensory receptors, inter- and motoneurons, and muscles and other peripheral organs (fat body, firefly lantern, salivary glands, corpora allata and corpora cardiaca, oviduct, etc.). Biogenic amines can initiate or modulate different types of behavior and they are involved in learning and the formation of memory in insects.

The effects of biogenic amines in the insect central nervous system are studied with the techniques of electrophysiological recordings, primary cell cultures, microinjections of amines and receptor ligands, and behavioral assays. Often the physiological responses to biogenic amines last for many minutes, which suggests that they can also act as neuromodulators. Biogenic amines modulate neuronal activity and the efficacy of synaptic transmission in all parts of the nervous system. The huge projection fields of many aminergic neurons support the idea of parallel modulation of entire neuronal circuits by just a few aminergic cells. In addition to synaptic neurotransmission, some aminergic neurons release the amine into the hemolymph. The substances are transported throughout the body and may thus have hormonal functions in specific target tissues.

The physiological role of OA at different levels of the organism is well documented. As a stress hormone in the periphery and in the central nervous system OA prepares the animal for energy-demanding behaviors. This monoamine stimulates glycogenolysis, modifies muscle contraction, supports long-term flight, and regulates "arousal" in the central nervous system. OA and OA agonists can enhance behavioral responses, like escape or aggressive behavior in crickets and sucrose responsiveness in honey bees. Injection of OA can elicit flight motor behavior in locusts, even in isolated thoracic ganglia. It is assumed that in insects OA has functions similar to those of the adrenergic system in vertebrates.

Both OA and 5-HT can modulate sensory receptors and receptor organs in insects. In many cases the sensitivities of the receptors are enhanced. Different funcions of OA and 5-HT at the sensory periphery are not very well understood, because the two amines often differ only in the degree of modulation. The increased sensitivity of sensory receptors due to the action of OA can modify behavior and is part of the "fight or flight" function. Studies on the *Drosophila* tyramine receptor mutant *hono* suggest that TA can also modulate the sensitivity of olfactory receptor cells, thus modulating behavioral responses to olfactory repellents.

The modulation of interneurons or effector neurons by biogenic amines is another level of modifying signal processing. OA and 5-HT can have functional antagonistic effects in a number of different systems. In these systems OA usually enhances the sensitivity or activity of single neurons and 5-HT usually has the opposite action. These effects, which can be measured at both the behavioral and the single-cell level, are dependent on the state of the insect. OA can induce a state of "arousal" in inactive animals and has only minor effects on very active animals, whereas 5-HT shows the largest effects in active animals.

In addition to modulatory functions during the adult life of an insect, DA and 5-HT have important functions during development. In *Drosophila*, high DA concentrations coincide with larval and pupal molts. Reduced levels of DA during larval stages lead to developmental retardation and decreased fertility in adults. 5-HT similarly acts as a chemical signal during larval development in *Drosophila*. Impaired 5-HT synthesis can lead to abnormal gastrulation movements, cuticular defects, and even embryonic death.

The neurotransmitter HA is released from photoreceptors in the compound eyes and ocelli in response to illumination. HA has also been detected in mechanosensory cells in *Drosophila*.

FUNCTIONS IN LEARNING AND MEMORY

Biogenic amines are involved in different forms of learning and memory formation in *Drosophila* and honey bees. However, it has not been unequivocally proven that the same biogenic amines serve identical functions in both species. Research on the neuronal and molecular bases of learning and memory over the past two decades in insects has focused on the mushroom bodies and antennal lobes of the brain. These two structures are involved primarily in processing of olfactory stimuli. Experimental evidence suggests that DA signals the presence of reinforcers and modulates intrinsic mushroom body neurons during conditioning in *Drosophila*. Thus DA could trigger signaling cascades that affect the storage of information about the conditioned stimulus.

In the honey bee, OA appears to be the modulatory transmitter which conveys information about rewarding sucrose stimuli and induces medium- to long-term modifications in interneurons during associative olfactory learning. Electrical stimulation of an identified octopaminergic cell, the ventral unpaired median VUM_{mx1} neuron, can substitute for the sucrose reward during olfactory conditioning. This neuron has extensive arborizations in different brain regions, including the antennal lobes and the mushroom bodies. Microinjections of OA into these two neuropiles of the bee brain confirmed that OA in fact induces associative learning.

See Also the Following Articles

Brain and Optic Lobes • *Chemoreception* • *Learning*

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Biogeographical Patterns

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Biogeography, which deals with the description and interpretation of plant and animal distributions, is linked with other sciences, especially ecology and (paleo-) geography; zoogeography is the branch addressing animal distribution.

Most animal species inhabit restricted ranges, and only relatively few are cosmopolitan. A comparison of the areas inhabited by different species reveals common distributional patterns that are complex reflections of the ecology and of active and passive animal dispersal, but also of the evolutionary history of both the species and the earth's surface. Zoogeography was sometimes divided into different disciplines, descriptive as opposed to causal zoogeography; the latter was then subdivided into ecological and historical zoogeography. Although studies may differ in their emphasis, the interrelations among these disciplines are too close for a formal division. This article describes the major zoogeographical patterns and uses selected examples from among the insects to highlight the significance of some of the factors just mentioned.

Insects are of great geological age, and most orders existed and were diverse when familiar vertebrates were only beginning to appear. Therefore, the distribution of most insect orders dates back much further than the distributions of many birds and mammals.

Insects are generally absent from some habitats. For example, with the exception of a few littoral specialists for unknown reasons, the only insects in the sea are some high ocean surface skaters among the bugs. Therefore, marine distribution patterns need not be considered here. The salt content of seawater is not the cause of this absence; insects are well represented in epicontinental waters of all kinds: fresh, brackish, and even hypersaline. Aquatic insects played an important role in the development of modern insect zoogeography. Because of their specific habitat ties, aquatic insects are easily collected, and the distributions of many are exceptionally well documented. Their distributions resemble those of terrestrial insects, in part because most aquatic insects have terrestrial adults that disperse over land.

The early explorers were struck by overall differences between the faunas of the lands they visited. The recognition of distinct faunal regions on a global scale thus has a long tradition and is briefly presented as an introduction. In addition to landmass topography, ecological conditions provide the basic setting for animal distributions; a brief outline of the major bioregions with similar overall ecology is therefore also presented.

Reproductively isolated species are the only naturally defined animal taxa; subspecific taxa can interbreed, whereas supraspecific taxa such as genera or families are human abstractions that change with conventions. It is convenient to use extant species to explain some concepts related to ranges and to discuss insect dispersal. Next, distribution patterns shaped by Pleistocene events are used to illustrate the importance of ecological change. The final focus is on disjunct (discontinuous, divided) distributions of monophyletic taxa that can best be explained by much older events, particularly continental drift.

ZOOGEOGRAPHICAL REGIONS

The major faunal regions (or realms) of the world only partly coincide with major landmasses (Fig. 1). Each region has a characteristic fauna distinguished by the particular combination of endemic taxa that exist in only this one region and those occurring also elsewhere. This early descriptive approach has long dominated zoogeography.

The Holarctic region is the largest region and is composed of the Palearctic and Nearctic regions, with many animals distributed over all the entire Holarctic region. Although a narrow land bridge (i.e., Central America) connects the Nearctic with the Neotropical region, the faunal change is pronounced. This land bridge is recent and was available only intermittently in the past. The Sahara Desert separates the Palearctic region from the Ethiopian (or Afrotropical) region, which includes the Arabian peninsula; Madagascar is now recognized as a distinct subregion. In Southeast Asia, climatic

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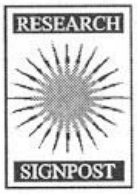

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14 Aminergic signal transduction in invertebrates: Focus on tyramine and octopamine receptors

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Abstract

Electro-chemical signal transduction is the basis of communication between neurons and their target cells. An important group of neuroactive substances that are released by action potentials from neurons are the biogenic amines. These are small organic molecules that bind to specific receptors located in the target cell membrane. Once activated these receptors cause changes in the intracellular concentration of second messengers, i.e. cyclic nucleotides, phosphoinositides, or Ca^{2+}, leading to slow but long-lasting cellular responses. Biochemical, pharmacological, physiological, and molecular biological approaches have unequivocally shown that biogenic amines are important regulators of cellular function in both vertebrates and invertebrates. In this review, we will concentrate on the properties of two biogenic amines and their receptors that were originally identified in invertebrates: tyramine and octopamine.

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Introduction

Some of the most fascinating properties of every neuron are to register, to evaluate, and to transmit information on a very fast time scale. Communication or, in molecular terms, signal transduction between neurons allows an organism to react to various external stimuli. Therefore, it is not surprising that changes in neural signaling efficacy can have a drastic impact on many physiological and behavioral processes including learning and memory. Most neurons in the central nervous system (CNS) use chemical synapses to convey information onto target organs or cells. An action potential stimulates the release of small organic compounds by exocytosis from specialized nerve terminals. These molecules may act as neurotransmitters, neuromodulators, or neurohormones.

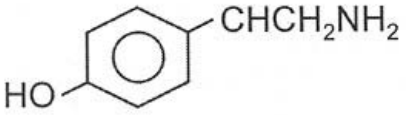
Neurotransmitters can mediate fast responses of the target cell. This usually occurs when neurotransmitters bind to receptors that form ion channels in the plasma membrane (ionotropic receptors). Well-known examples for fast-acting neurotransmitters are acetylcholine and glutamate, which cause the excitation of neurons, whereas glycine and γ-aminobutyric acid (GABA) cause the inhibition of neurons. One group of neuroactive substances that primarily act by binding to membrane proteins that themselves do not form ion channels are the biogenic amines. These molecules are derived from amino acids by biochemical modification. In vertebrates, five compounds are considered as classical biogenic amines: dopamine, norepinephrine, epinephrine, serotonin (5-hydroxytryptamine, 5-HT), and histamine. Whereas dopamine, 5-HT, and histamine are also present in invertebrates, norepinephrine and epinephrine seem to be functionally substituted by two structurally related compounds: p-tyramine and octopamine [1, 2, 3]. Very low levels of p-tyramine and octopamine and of tryptamine and β-phenylethylamine have also been identified in mammals. In the mammalian brain, these substances account for less than 1% of all biogenic amines. Because of their low abundance, they are collectively called "trace amines" and have long been thought of as metabolic by-products only [4, 5]. However, the level of trace amines has been found to be altered in various human disorders (including depression, hepatic encephalopathy, hypertension, Parkinsonism, phenylketonuria, and schizophrenia) suggesting that these substances are physiologically relevant [6].

With a few exceptions, viz., the 5-HT_3 receptor channel in vertebrates [7] and histamine-gated ion channels in invertebrates [8, 9], all known biogenic amine receptors belong to the superfamily of G protein-coupled receptors (GPCRs) [10, 11]. Since the activation of GPCRs usually leads to metabolic changes in the target cell, they are also called metabotropic receptors. Ligand binding to GPCRs induces conformational changes of the protein and thereby activates the receptor. Activated GPCRs usually transmit the signal to intracellular trimeric GTP-binding (G) proteins. The activated G proteins can either alter the opening probability of ion channels or the activity of specific target enzymes. Physiologically, the activation or inhibition of these enzymes leads to changes in the intracellular concentration of cyclic nucleotides (cAMP, cGMP), inositol-1,4,5-trisphosphate (IP_3), diacylglycerol (DAG), or arachidonic acid metabolites (eicosanoids). Subsequently, the activity of second messenger-dependent enzymes changes thus causing modification of the functional properties of various cytosolic, membrane-bound, and nuclear proteins.

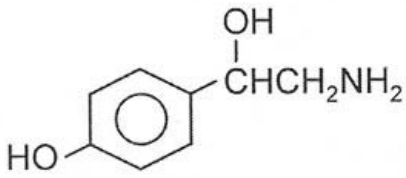
In this review, we will describe the properties of tyramine and octopamine receptors that initially were thought to be almost exclusively expressed in invertebrates. The recent isolation of mammalian trace amine receptor genes [12, 13], however, strongly suggests a role for tyramine and other trace amines as bona fide neurotransmitters also in vertebrates. We are going to compare the properties of known tyramine and octopamine receptors on the biochemical, pharmacological, molecular biological, and cellular signaling level. The interested reader will find other information on biogenic amines and their respective receptor systems not covered by this article in a number of recent and more comprehensive reviews [3, 11, 14 - 20].

Biosynthesis of tyramine and octopamine

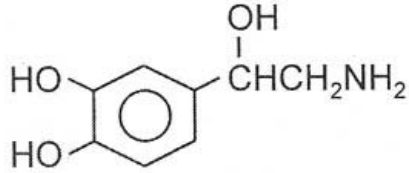
The precursor of the phenolamines tyramine and octopamine is the essential amino acid L-tyrosine. Direct decarboxylation of tyrosine by tyrosine decarboxylase generates the phenolamine tyramine. For a long time, tyramine was considered to be solely an intermediate reaction product of the synthesis of octopamine. This assumption was based on the finding that octopamine is derived from tyramine by hydroxylation on the β-carbon of the side chain (Fig. 1). This reaction is catalyzed by the enzyme tyramine



tyramine



octopamine



norepinephrine

Figure 1. Structural formulae of p-tyramine, octopamine, and norepinephrine.

β-hydroxylase, an enzyme the gene of which has been cloned in *Drosophila* [21]. The phenolamine octopamine and the catecholamine norepinephrine share substantial structural similarity (Fig. 1). They only differ in a hydroxyl group in the *meta* position of the benzoyl moiety of norepinephrine. Therefore, it is assumed that the norepinephrine/ epinephrine system of vertebrates is functionally substituted by the tyraminergic/ octopaminergic system in invertebrates [1, 2, 3].

Molecular properties of tyramine and octopamine receptors

Biogenic amines such as tyramine and octopamine bind to receptor proteins that are derived from a superfamily of related genes. Collectively these receptors are called GPCRs. Biogenic amine receptors are members of a receptor subfamily, of which rhodopsin was the founder. Based on recent crystal structure data [22, 23], hydropathy analyses of the deduced amino acid sequences, and phylogenetic comparisons [10, 24 - 27], GPCRs share the common motif of seven transmembrane (TM) domains (Fig. 2). The N-terminus is located extracellularly and the C-terminus is located intracellularly. The N-terminus is the target for post-translational modification and often contains consensus sequence motives for N-linked glycosylation [28, 29]. The membrane-spanning regions are linked by three extracellular loops (EL) that alternate with three intracellular loops (IL). Cysteine residues in the cytoplasmic tail of the polypeptides are the targets of post-translational palmitoylation. Insertion of these fatty acids into the plasma membrane will create a fourth intracellular loop (IL4) and is assumed to stabilize the structure of these receptors [30].

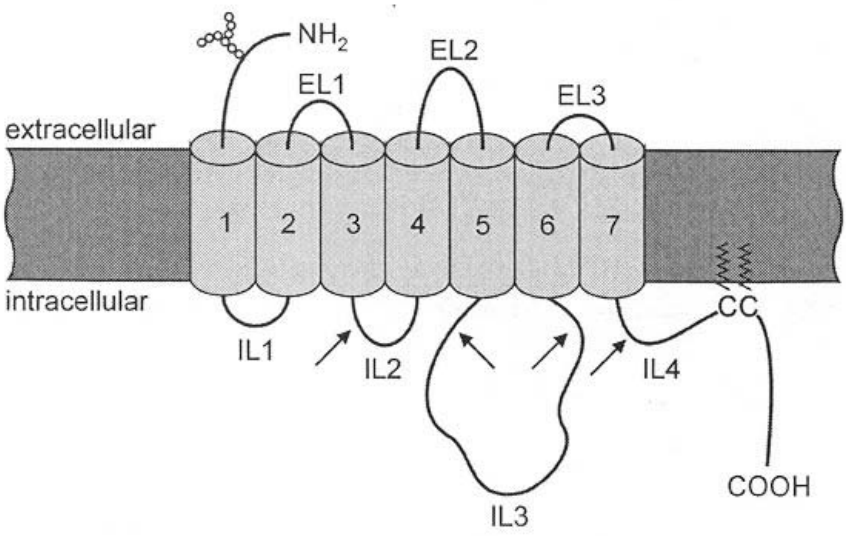


Figure 2. Topography of a biogenic amine receptor. The polypeptide spans the membrane seven times. The transmembrane regions (TM 1-7) are depicted as cylinders. The N-terminus (NH_2) is located extracellularly and often contains glycosylated residues (o). The C-terminus (COOH) is located intracellularly. The membrane spanning regions are linked by three extracellular loops (EL) that alternate with three intracellular loops (IL). When the protein is post-translationally palmitoylated at cysteine residues (C) in the cytoplasmic tail, a fourth intracellular loop (IL4) is formed. Arrows point to receptor segments involved in G protein binding.

Activation of the receptor occurs by the binding of a biogenic amine. This interaction takes place in a binding pocket formed by the TM regions in the plane of the membrane [31]. Specific residues in different TM segments interact with functional groups of the biogenic amines. Tyramine and octopamine receptors contain highly conserved amino acid residues originally identified as ligand-binding partners in other biogenic amine receptors. In particular, an aspartic acid residue in TM3, serine residues in TM5, and a phenylalanine residue in TM6 that have been shown to determine the ligand-binding properties of many biogenic amine receptors [26, 29] are also present in tyramine and octopamine receptor sequences. Once the ligand is tightly bound to its receptor, the conformation of the receptor will change [32]. This structural change is usually transferred to trimeric G proteins [27, 33]. Residues of IL2, 3, and 4 in close vicinity to the plasma membrane determine the specificity and efficacy of G protein activation (Fig. 2). Once activated, G protein α- and $\beta\gamma$-subunits regulate the activities of a structurally diverse group of effector molecules. These include enzymes participating in the synthesis and degradation of intracellular second messengers and ion-selective channels. Adenylyl cyclases often become activated or inhibited leading to changes in the intracellular concentration of cyclic AMP ($[cAMP]_i$). Another important pathway leads to the activation of phospholipase C (PLC). This enzyme generates the second messengers IP_3 and DAG, which cause Ca^{2+} release from intracellular stores and activation of protein kinase C (PKC), respectively. Receptor-mediated signaling can be turned off by the phosphorylation of serine and threonine residues in the C-terminus and IL3 of the receptor protein and by β-arrestin binding [34 - 37]. In addition to these classical signaling pathways, some recent observations strongly suggest that GPCRs also transduce signals by G protein-independent mechanisms (for reviews, see: [38, 39]).

The first tyramine receptor cDNA was independently cloned by two groups in 1990 from *Drosophila melanogaster* [40, 41]. Since then, a large number of related genes from various invertebrates and, recently, also from mammalian species have been cloned or deposited in sequence databases. We have used the *Drosophila* (DmTYR [41]) and *Apis mellifera* (AmTYR1 [42]) tyramine receptor sequences as "baits" to search the GenBank database for homologous genes. In addition, we also used the sequence of an octopamine receptor from *Drosophila* (OAMB [43]) to identify octopamine receptor genes. The results of these screens are summarized in Table 1 and in a dendrogram (Fig. 3) to illustrate the phylogenetic relationship. For convenience, we have included a dopamine receptor from *Drosophila* (DmDOP1 [51]), a serotonin receptor from *Drosophila* (Dm5HT7 [52]), and the human α_{2B}-adrenergic receptor (hA2B [53]) in these trees.

Based on these analyses, tyramine and octopamine receptors have been placed into separate clades within the biogenic amine receptor subfamily of GPCRs. Both groups are separated by the serotonin (Dm5HT7) and the α-adrenergic receptor (hA2B) sequences. Notably, two mammalian trace amine receptor sequences are only distantly related to all other sequences analyzed (Fig. 3). Although a considerable number of tyramine/ octopamine receptor sequences can be obtained from the databases, so far only a few of these genes are functionally expressed and characterized in terms of their pharmacology and second messenger coupling (for a review, see: [11]).

Table 1. Search of NCBI database with Amtyr1, Dmtyr, and OAMB receptor sequences for homologous genes and proteins

query	identified gene	accession number nucleic acid	accession number amino acid	e-value nucleic acid	e-value amino acid	reference
Dmtyr	*Drosophila melanogaster* Dmtyr	X54794	S12004	0.0	0.0	[40, 41]
	Bombyx mori BomOA1	X95607	Q17232		e-111	[44]
	Locusta migratoria Loctyr2	X69521	Q25322	2e-48	3e-70	[45]
	Locusta migratoria Loctyr1	X69520	Q25321	2e-43	3e-70	[45]
	Heliothis virescens HelOA1	X95606	Q25188		1e-68	[44]
	Balanus amphitrite Baltyr2	D78363	Q93127		8e-56	[46]
	Balanus amphitrite Baltyr1	D85547	Q93126		3e-31	[47]
	Boophilus microplus Bootyr1	AJ010743	CAA90335	3e-23		[48]
	Lymnea stagnalis LymOA1	U62771	AAC61296		3e-23	[49]
	Lymnea stagnalis LymOA2	U62770	AAC16969		2e-22	[50]
	Apis mellifera Amtyr1	AJ245824	CAB76374	2e-15		[42]
	Mamestra brassicae Mamtyr	AF343878	AAK14402	2e-11		
Amtyr1	*Apis mellifera* Amtyr1	AJ245824	CAB76374	0.0		[42]
	Balanus amphitrite Baltyr2	D78363	Q93127		8e-94	[46]
	Locusta migratoria Loctyr2	X69521	Q25322	1e-19	2e-77	[45]
	Locusta migratoria Loctyr1	X69520	Q25321	1e-17	3e-77	[45]
	Drosophila melanogaster Dmtyr	X54794	P22270	2e-15	5e-75	[40, 41]
	Bombyx mori BomOA1	X95607	Q17232		5e-74	[44]
	Heliothis virescens HelOA1	X95606	Q25188		2e-73	[44]
	Balanus amphitrite Baltyr1	D85547	Q93126		3e-36	[47]
	Lymnea stagnalis LymOA2	U62770	O01670		7e-28	[50]
	Boophilus microplus Bootyr1	AJ010743	CAA90335	8e-12		[48]
OAMB	*Drosophila melanogaster* OAMB	AF065443	AAC17442	0.0		[43]
	Balanus amphitrite Baltyr1	D78363	Q93126	6e-5	6e-50	[47]
	Lymnea stagnalis LymOA1	U62771	AAC61296	6e-5	4e-39	[49]
	Locusta migratoria Loctyr1	X69520	Q25321	0.25	2e-28	[45]
	Locusta migratoria Loctyr2	X69521	Q25322	0.25	2e-28	[45]
	Drosophila melanogaster Dmtyr	X54794	P22270		4e-28	[40, 41]
	Heliothis virescens HelOA1	X95606	Q25188		2e-27	[44]
	Bombyx mori BomOA1	X95607	Q17232		1e-26	[44]
	Balanus amphitrite Baltyr2	D78363	Q93127		6e-26	[46]
	Lymnea stagnalis LymOA2	U62770	O01670		2e-20	[50]

Functional properties of cloned tyramine and octopamine receptors

Functional characterization of cloned genes requires expression of the gene in heterologous expression systems. A variety of suitable expression vectors and established cell lines are available to perform these tests. Since different laboratories favor different expression systems, this diversity probably has an impact on the results obtained. In Table 2, we have summarized some of the characteristic features described for heterologously expressed tyramine and octopamine receptors.

What conclusions can be drawn from the above-mentioned experimental data? Most of the heterologously expressed tyramine receptor genes from invertebrate species encode GPCRs that inhibit cAMP production in cells. Therefore, the activated receptors probably bind to and stimulate G_i-type G proteins [27, 59, 60]. Since this coupling occurs in various cell lines from vertebrates (e.g., HEK 293, NIH 3T3, CHO) and non-vertebrate (e.g., Sf9, *Drosophila* S2) origin (Table 2), tyramine receptor activation *in vivo* will probably also cause the attenuation of adenylyl cyclase activity. We have previously mentioned that the NH_2-terminal and COOH-terminal extremes of the IL3 of the receptors appear to play a prominent role in G protein interaction (Fig. 2). An amino acid sequence comparison of these regions for some tyramine receptors is depicted in Figure 4. The highest degree of sequence conservation is seen between receptors from

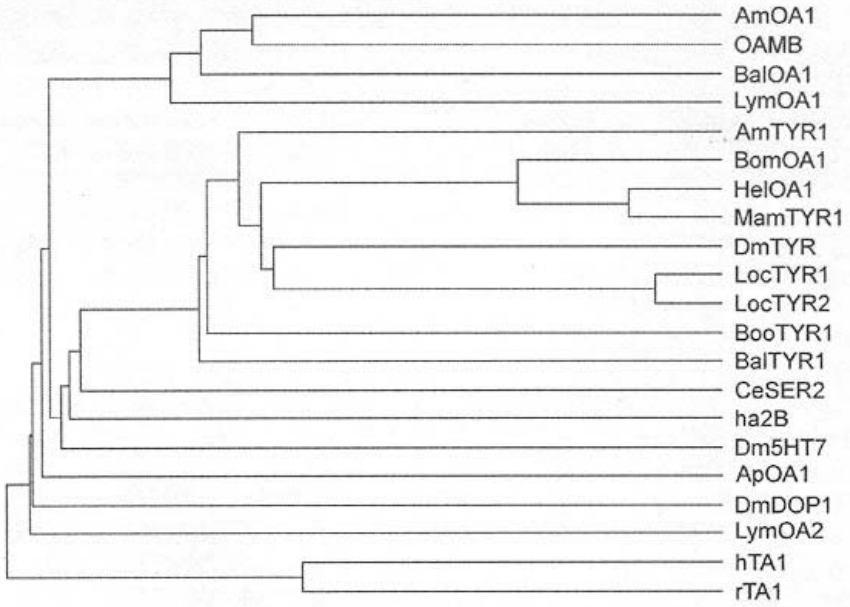


Figure 3. Dendrogram of biogenic amine receptor sequences. Sequence alignment was performed with the CLUSTAL program of PCGENE software (version 6.6; IntelliGenetic) and the complete amino acid sequence of each receptor. The receptor sequences followed by their GenBank accession numbers (#) are listed in the order illustrated: *Apis mellifera* octopamine (AmOA1, #CAD67999), *Drosophila melanogaster* octopamine (OAMB, #AAC17442), *Balanus amphitrite* octopamine (BalOA1, #Q93127), *Lymnea stagnalis* octopamine 1 (LymOA1, #AAC61296), *A. mellifera* tyramine (AmTYR1, #CAB76374), *Bombyx mori* "octopamine" (BomOA1, #Q17232), *Heliothis virescens* "octopamine" (HelOA1, #Q25188), *Mamestra brassicae* tyramine (MamTYR1, #AF343878), *D. melanogaster* tyramine (DmTYR, #P22270), *Locusta migratoria* tyramine (LocTYR1, #X69520; LocTYR2, #X69521), *Boophilus microplus* tyramine (BooTYR1, #AJ010743), *B. amphitrite* tyramine (BalTYR1, #Q93126), human α_{2B}-adrenergic (hA2B, #NP_000673), *D. melanogaster* serotonin (Dm5HT7, #P20905), *Aplysia kurodai* octopamine (ApOA1, #AAF28802), *D. melanogaster* dopamine (DmDOP1, #CAA54451), *L. stagnalis* octopamine 2 (LymOA2, #O01670), human trace amine receptor 1 (hTA1, #AAK71236), and rat trace amine receptor 1 (rTA1, #AAK71237).

Drosophila (DmTYR [41]), *Apis* (AmTYR1 [42]), *Locusta* (LocTYR1 [45]), and *Heliothis* (HelOA1 [44]). In these regions, ≥95% of the residues are identical or conservatively substituted. A common characteristic of these receptors is that they attenuate adenylyl cyclase activity. A receptor recently cloned from *Caenorhabditis elegans* (CeSER2 [57]) also inhibits cAMP production in response to tyramine application. It shares ~80% sequence similarity with DmTYR. Sequence conservation, however, is less pronounced between those receptors that have been found to activate different intracellular signaling pathways. Both the rat and human trace amine receptors (rTA1 and hTA1 [12]) stimulate adenylyl cyclase, whereas the *Drosophila* octopamine receptor (OAMB [43]) causes an increase in $[Ca^{2+}]_i$ and $[cAMP]_i$. These receptors share only 43% - 63% of amino acid residues with DmTYR (Fig. 4) supporting the assumption that the amino acid residues flanking TM5 and TM6 largely determine the functional coupling properties of the receptors.

Table 2. Intracellular signaling pathways activated by heterologously expressed tyramine and octopamine receptors.

receptor	species	expression system	intracellular signalling	agonist preference	reference
AmTYR1	*Apis mellifera*	HEK 293	cAMP↓	tyr > oct	[42]
DmTYR	*Drosophila melanogaster*	NIH 3T3	cAMP↓	tyr > oct	[41]
		CHO-K1	cAMP↓	tyr > oct	[54]
			Ca^{2+}↑	oct ≥ tyr	
		Xenopus oocyte	Ca^{2+}↑	oct ~ tyr	[55]
LocTYR1	*Locusta migratoria*	Sf9, S2	cAMP↓	tyr > oct	[45]
		MEL-C88L	Ca^{2+}↑ and cAMP↓	tyr > oct	[56]
CeSER2	*Cenorhabditis elegans*	HEK 293	cAMP↓	tyr > oct	[57]
rTA1	rat	*Xenopus* oocyte	cAMP↑/Cl^- current	tyr > oct	[12]
		HEK 293	cAMP↑	tyr > oct	[13]
hTA1	human	*Xenopus* oocyte	cAMP↑/Cl^- current	tyr > oct	[12]
		COS-7	cAMP↑	tyr > oct	
OAMB	*Drosophila melanogaster*	HEK 293, S2	cAMP↑ and Ca^{2+}↑	oct > tyr	[43]
LymOA1	*Lymnea stagnalis*	HEK 293	cAMP↑ and Ca^{2+}↑	oct > tyr	[49]
LymOA2	*Lymnea stagnalis*	HEK 293	Cl^- current↑	oct > tyr	[50]
ApOA1	*Aplysia californica*	HEK 293, CHO	cAMP↑	oct > tyr	[58]
		Xenopus oocyte	cAMP↑/Cl^- current	tyr > oct	
HelOA1	*Heliothis virescens*	LLC-PK_1	cAMP↓	oct > tyr	[44]

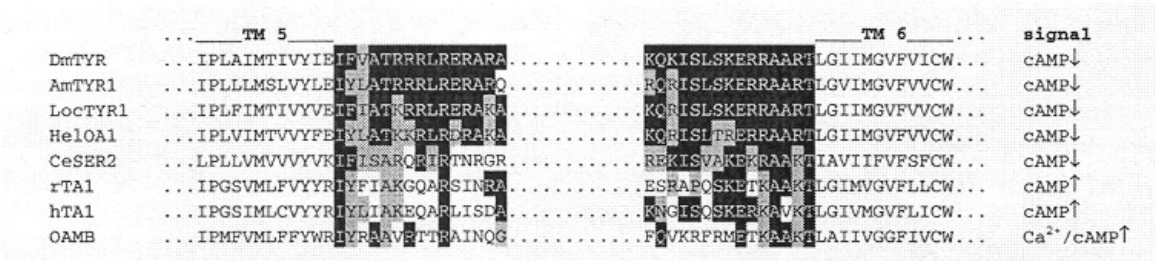


Figure 4. Sequence alignment of residues flanking TM5 and TM6 in tyramine receptors. The alignment shows part of the TM5 and TM6 segments. Fifteen amino acid residues flanking each of these TMs in the intracellular loop (IL3) are displayed. Identical residues in IL3 are given as white letters against black, conservative substitutions are highlighted in gray. The alignment was performed with tyramine receptor sequences from: *Drosophila* (DmTYR, #P22270), *Apis* (AmTYR1, #CAB76374), *Locusta* (LocTYR1, #X69520), *Heliothis* (HelOA1, #Q25188), *Caenorhabditis* (CeSER2 [57]), rat (rTA1, #AAK71237), human (hTA1, #AAK71236), and an octopamine receptor from *Drosophila* (OAMB, #AAC17442). The signaling pathways activated by the receptors are given on the right.

The recent cloning of mammalian GPCRs that are activated by trace amines [12, 13] adds at least two additional members to the tyramine receptor family, rat trace amine receptor 1 (rTA1) and human trace amine receptor 1 (hTA1). Both are more potently activated by tyramine than by octopamine [12, 13]. The signaling properties of the receptors were tested after injection of receptor mRNAs, together with mRNA encoding a cAMP-responsive Cl^- channel (CFTR), into *Xenopus* oocytes. Receptor activation by trace amines (tyramine and β-phenylethylamine) resulted in a Cl^- inward current. This result suggests that the mammalian TA1 receptors cause the stimulation of adenylyl cyclase through G_s-type G proteins. The increase in $[cAMP]_i$ leads to CFTR activation and Cl^- conductance. In comparison with invertebrate sequences, both rTA1 and hTA1 receptors contain more variant residues in IL3 (Fig. 4). This might be the reason for their different signaling properties.

What information can be drawn from the expression studies of octopamine receptor genes? The octopaminergic system of invertebrate species has attracted much more interest than the tyraminergic signaling system, and the physiological role of octopamine has been studied *in vivo* extensively (for recent reviews, see: [3, 11, 61]. Pharmacologically, four different types of octopamine receptor have been distinguished. They are associated either with an increase in $[Ca^{2+}]_i$ (OCT-1 receptor) or with the activation of adenylyl cyclases (OCT-2A, OCT-2B, and OCT-3 receptors) [1, 3, 62 - 67]. Molecular cloning of octopamine receptors has lagged behind the molecular identification of other biogenic amine receptors. The first *Drosophila* gene cloned and claimed to encode an octopamine receptor [40] was convincingly shown to encode a tyramine receptor (DmTYR, see above and [41]). The DmTYR receptor attenuates cAMP production. Related receptor genes have been cloned from moths, *Heliothis virescens* and *Bombyx mori* (HelOA1 and BomOA1 [44]). The heterologously expressed HelOA1 receptor causes inhibition of adenylyl cyclase activity when stimulated with octopamine, whereas no significant effect is observed with tyramine [44]. This result does not agree with the known signaling properties of native octopamine receptors. So far, only increases in $[cAMP]_i$ and/or $[Ca]_i$ have been described for octopamine receptors *in vivo* [68], in intact organ preparations [62-64], in membrane preparations of various tissues [42, 69 - 72], and in various insect cell lines [73 - 77]. Interestingly, the receptors of both moths share significant sequence identity with tyramine receptors. Residues involved in G protein binding are almost identical between HelOA1, BomOA1, DmTYR, AmTYR1, and LocTYR1 (Fig. 4). One thus might consider re-evaluating the pharmacological properties of the *Heliothis* receptor with respect to agonist affinity and efficacy.

In the meantime, however, one receptor from *Drosophila* (OAMB [43]), one from the sea slug *Aplysia californica* (ApOA1 [58]), and one from the pond snail *Lymnea stagnalis* (LymOA1 [49]) have been found to be specifically activated by octopamine. The signaling properties of these receptors largely confirm what is known from native receptors. The receptor cloned from *Aplysia* exclusively stimulates adenylyl cyclase activity [58]. Two cellular signals are produced by both the *Drosophila* and the *Lymnea* receptors. When activated by octopamine, they cause an elevation of $[cAMP]_i$ and $[Ca^{2+}]_i$ [43, 49]. The promiscuous coupling to both intracellular signaling pathways might be attributable to the expression system chosen, and it remains to be tested to which signaling pathway these two receptors couple *in vivo*. Our own results obtained

for an octopamine receptor from *Apis mellifera* (AmOA1 [78]) show that this receptor specifically activates the IP_3/Ca^{2+} pathway. Since AmOA1 is orthologous to LymOA1 and OAMB, one might assume these receptors form the native OCT-1 receptor class that is known to cause Ca^{2+} elevation in cells [1, 3, 67].

Meanwhile, a second octopamine receptor (LymOA2) cDNA has been cloned from *Lymnaea stagnalis* [50]. This receptor seems to couple to an atypical signaling pathway. Stimulation of heterologously expressed LymOA2 with octopamine leads to changes in the membrane conductance of the cells. It has been proposed that the receptor activates a voltage-independent Cl^- current by a mechanism that probably involves protein phosphorylation [50]. Whether stimulation of LymOA2 in *Lymnaea* neurons *in vivo* also affect Cl^- conductances remains to be investigated.

Physiological functions of tyraminergic and octopaminergic systems *in vivo*

In comparison with vertebrates in which only trace amounts of tyramine and octopamine have been detected, both phenolamines are present in high concentrations in the CNS and periphery of invertebrates [1, 5, 79, 80]. It was assumed for a long time that tyramine only serves as a biochemical precursor of octopamine and thus might not have any significant neuroactive function. However, molecular cloning and the functional characterization of specific tyramine receptors from various invertebrate species [41, 42, 45, 57] and recently also from rat and human [12, 13] call for a re-evaluation of the primary assumptions. Nevertheless, the physiological role of the tyraminergic system *in vivo* needs to be examined by further studies. Only a few reports have been published addressing the functional role of tyramine. Tyramine has been shown to alter the behavioral sensitivity of *Drosophila* to cocaine [81]. Exposure of flies to cocaine leads to an increase in tyrosine decarboxylase activity, which parallels the development of sensitization. In addition, tyramine causes an increase in chloride conductance across the *Drosophila* Malpighian tubule [82]. Physiological effects specific for tyramine have also been reported for other insects. In the cockroach, tyramine stimulates trehalose metabolism in isolated fat bodies [83]. In addition to fulfilling important criteria as a transmitter candidate in the locust CNS [84], tyramine inhibits the contraction of locust visceral muscles [85] and, after repeated injections, tyramine even reduces locust viability [86].

Analysis of biogenic amine signaling systems in invertebrates suffers from the lack of receptor mutants. This situation has recently been overcome. A tyramine receptor mutant with reduced receptor expression levels has been identified in *Drosophila* [87]. The *hono* mutant displays deficits in olfactory responses. Furthermore, tyramine fails to inhibit excitatory junctional potentials at larval body-wall muscles in the mutant. Tyramine is considered to be well-suited to act as a neuromodulator in the olfactory system and at the neuromuscular junction [87, 88]. However, tyramine and octopamine are structurally very similar, and tyramine receptors have been shown to be activated by octopamine, although at significantly higher concentrations, in pharmacological experiments and *vice versa* [41, 42, 43]. One could assume that some of the effects attributed to the missing tyramine receptors might be caused by promiscuously activated octopamine receptors. How could this take place? In *hono* flies, tyramine synthesis is not

impaired. The endogenous binding partner of tyramine, the receptor encoded by the *hono* gene, however, is almost absent. When tyramine is released upon activation of tyraminergic neurons, this might lead to an unusually high concentration of this amine. Since its ordinary binding partner is absent, tyramine instead might bind to octopamine receptors. These octopamine receptors are coupled to different intracellular signaling pathways from tyramine receptors. Rather than inhibiting cAMP production, a common property of tyramine receptors, octopamine receptors cause the production of cAMP or IP_3 and subsequent Ca^{2+} release. Because of these unusual actions of tyramine, the highly controlled neuronal circuits thus affected will be disturbed and cause the observed impairments. This interpretation is however speculative and needs to be tested by experimental approaches.

In contrast to tyramine, the physiological roles of octopamine have been studied in great detail in a number of invertebrate species. Octopamine can act as a neurotransmitter, a neuromodulator, and a neurohormone (for recent reviews, see: [3, 14, 15, 89]). Specific effects of octopamine have been described for both the CNS and peripheral organs. In insects, octopamine is referred to as a "flight or fight" hormone or a "sympathetic" circulation hormone [1, 2, 3, 5, 67, 79]. How can one address the role of a neuroactive substance when receptor mutants are missing? In a straightforward approach, Monastirioti et al. [21] have used a *Drosophila* mutant that does not express the enzyme tyramine-ß-hydroxylase. This enzyme is necessary to convert tyramine to octopamine. Therefore, these flies cannot synthesize octopamine. Nevertheless, the animals exhibit almost normal behavior and life span. The only phenotypic change occurs in female flies. They are unable to deposit their eggs properly and are therefore sterile. Sterility is overcome, however, by feeding them octopamine [21]. Although these results suggest that octopamine is not necessary for the proper development and differentiation of the fly, it is currently unclear whether the loss of octopamine might have been functionally substituted by promiscuous binding of tyramine to octopamine receptors.

Octopamine plays an important role during insect flight. This has been intensively studied in locusts (for reviews, see: [61, 90]). Previous work has suggested that octopamine influences the energy metabolism of flight muscles. It increases the concentration of fructose-2,6-bisphosphate and stimulates glycolysis [91 - 94]. During long flight periods, the flight muscles switch to lipid metabolism as the energy source [93, 94]. A subpopulation of octopaminergic dorsal unpaired median (DUM) neurons, viz., those that exclusively innervate flight power muscles, are inhibited during short bouts of flight activity [95]. It is assumed that the decrease in octopaminergic neurotransmission switches off the glycolytic pathway and thereby turns on lipid metabolism [61, 95]. Another group of DUM neurons, which activate leg and other thoracic muscles, behave differently in that they are activated during flight activity [95]. Leg muscles lack the biochemical machinery for lipid metabolism. Therefore, glycolysis has to be maintained for energy production in these muscles even during flight [95].

Skeletal and visceral muscles are the main targets for the octopaminergic efferent DUM neurons. Modulation of neuromuscular transmission by bath application of octopamine and/or stimulation of individual DUM neurons has been studied in great detail (for recent reviews, see: [61, 96]). Octopamine reduces basic tension and causes an increase in twitch amplitudes and relaxation rates in skeletal muscles [97 - 101]. It modulates heart rate in *Drosophila* [102, 103] and inhibits myogenic rhythms of visceral

muscles [104-106]. Generally, the octopaminergic system of invertebrates, like the norepinephrine/epinephrine system of vertebrates, seems to adapt the animals to energy-demanding situations ("fight or flight") [3]. In addition, octopamine also influences the response characteristics of sensory organs, including mechanoreceptors [107-110] and pheromone receptors [111, 112].

In a variety of behavioral tests, the effects of various amines have been examined by bath application, feeding, or injection. Response thresholds and habituation rates of feeding responses in honeybees [113-115] and flies [116] and of visual responses in bees [117, 118] and locusts [89, 119, 120] are significantly reduced by octopamine. Furthermore, octopamine modulates antennal scanning in the bee [121]. In the honeybee sting response, octopamine reduces the rhythmic motor component of the reflex but potentiates sting extension [122]. Similar differential effects have also been observed in the lobster, where octopamine selectively enhances the activity of neurons and muscles responsible for generating the "extension posture", while diminishing the activity in those causing the antagonistic "flexion posture" [123-125]. Other behaviors that are induced or modulated by octopamine are pharyngeal pumping, locomotion, and egg-laying in *Caenorhabditis elegans* [126], firefly flashing [127], locomotory and grooming patterns in decapitated *Drosophila* [128], and nestmate recognition and the onset of foraging in honeybees [129-131].

One particular octopaminergic neuron, VUM_{mx1}, plays an important role in the reinforcement pathway during honeybee olfactory conditioning [132]. The VUM_{mx1} neuron depolarizes in response to the presentation of sucrose rewards to antennae and proboscis. Current injection into the VUM_{mx1} neuron or octopamine injection into either the antennal lobe or the calyces of the mushroom bodies can substitute for the sucrose reward during olfactory conditioning [133]. It has been concluded that octopamine selectively mediates the reinforcing but not the sensitizing or response-releasing function of the sucrose reward [134]. Recently, Farooqui et al. [135] have used the RNA interference (RNAi) technique to disrupt the octopamine-mediated reinforcement pathway involved in honeybee olfactory learning. Injection of RNAi directed against a putative octopamine receptor gene into the antennal lobe resulted in 80% and 50% inhibition of acquisition and recall responses, respectively [135]. This is a very promising result because it shows that the RNAi technique is a suitable tool for studying impaired gene function(s) in a behaviorally well characterized organism that is, however, difficult to manipulate genetically. We expect that, in the near future, the application of RNAi techniques will shed more light on the functions of invertebrate octopamine and tyramine receptors, which may include the possible involvement in learning and memory as one particularly fascinating aspect.

Conclusion

The phenolamines tyramine and octopamine are important mediators and regulators of diverse physiological functions in invertebrates and probably also in vertebrates. A prerequisite to understanding the physiological role of the tyraminergic and octopaminergic signaling systems is the molecular characterization of the respective receptors. Once the receptor genes are cloned, heterologous expression should aid the identification of their downstream reaction partners. The application of molecular biological methods has greatly enhanced our knowledge of the receptor polypeptides.

From these studies and the analysis of completely sequenced genomes of the nematode *Caenorhabditis elegans* [136] and various insects (*Drosophila melanogaster* [17], *Anopheles gambiae* [137]), it has become evident that multiple tyramine and octopamine receptor subtypes exist in invertebrates. All receptors identified so far belong to the superfamily of GPCRs. Heterologously expressed receptors usually activate the same signaling pathways as native receptors *in vivo*. Nevertheless, several receptor subtypes still appear to be "missing" as not all of the pharmacological and signaling properties of octopamine and tyramine receptors described *in vivo* are covered by the cloned receptors. However, this gap will certainly soon be closed for some "model organisms" by detailed analysis of their completely sequenced genomes. Knowledge of the receptor sequences and the most probable signaling partners of the various receptors should then lead to the design of experimental approaches aimed at elucidating the functions of the receptors *in vivo*. For some organisms, RNAi may become a versatile technical tool, and we eagerly anticipate results indicating whether the concepts that have been extrapolated from heterologous systems also apply *in vivo*.

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Preface

Cellular Actions of Biogenic Amines

Wolfgang Blenau*

Biogenic amines are important messenger substances and regulators of cell function. In insects, these small organic compounds act as neurotransmitters, neuromodulators, and neurohormones (for reviews, see: Evans, 1980; Downer, 1990; Roeder, 1994; Blenau and Baumann, 2001, 2003; Baumann et al., 2003; Roeder et al., 2003). Biogenic amines control endocrine and exocrine secretion (Just and Walz, 1996; Marg et al., 2004), the contraction properties of muscles, the activity of neurons (for a review, see Bicker and Menzel, 1989), and the generation of motor patterns (Claassen and Kammer, 1986). In addition, certain biogenic amines are involved in learning and the formation of memory (Schwaerzel et al., 2003; for reviews, see: Meller and Davis, 1996; Hammer, 1997). Biogenic amines mediate these diverse cellular and physiological effects by binding to G protein–coupled receptors (GPCRs; for reviews, see: Vernier et al., 1995; Blenau and Baumann, 2001, 2003; Park and Adams, 2005), which are integral membrane proteins that sense and transduce extracellular signals into specific cellular responses.

Although much is known about the biological role of the biogenic amines dopamine, tyramine, octopamine, serotonin, and histamine in the insect nervous system, our knowledge of the molecular and functional properties of insect GPCRs is rather limited. In recent years, however, considerable progress has been made toward an understanding of the functional properties of these receptors (and of amine transporters) at the cellular and molecular level. The availability of completely sequenced insect genomes (i.e., *Drosophila melanogaster, Anopheles gambiae*, and *Apis mellifera*) provides excellent tools to unravel the various families of insect biogenic amine receptors in detail (Brody and Cravchik, 2000; Vanden Broeck, 2001; Hill et al., 2002). Pioneering work on *Drosophila* receptors is now being followed by molecular studies in other species of insects that are well suited for physiological and behavioral experiments. This special issue on "Cellular Actions of Biogenic Amines" covers recent research developments in insect biogenic amine research with a focus on "larger" insect species.

The first report in this issue is a review providing a comprehensive overview of the present knowledge of invertebrate dopamine receptors (Mustard et al., 2005, this issue). It focuses on results gained from *Apis mellifera, Drosophila melanogaster,* and *Caenorhabditis elegans*, renowned model organisms for behavioural and genetic analyses. The molecular properties of three groups of invertebrate dopamine receptors identified so far (i.e., the "DOP1"-, the "invertebrate-type," and the D2-like dopamine-receptors) are summarized and their functional implications are discussed. The second report describes the functional properties of the promoter region of a "DOP1"-type dopamine receptor, i.e., the *Drosophila* DmDOP1 receptor (Kehren and Baumann, 2005, this issue). RACE-

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PCR and primer-extension techniques have been applied to identify the transcriptional start site of the gene. Promoter activity has been assessed by CAT reporter-gene assays.

Molaei and co-workers (2005, this issue) report the cloning of a putative GPCR cDNA, grc3, from visceral muscle of the locust, *Locusta migratoria*. Interestingly, the grc3 sequence shows a high degree of sequence conservation to a tyramine receptor cDNA previously cloned from the nervous system of *Locusta* (gcr1, Vanden Broeck et al., 1995). Thus, grc3 most probably encodes a second tyramine receptor expressed in the locust (Molaei et al., 2005, this issue). The following two reports provide detailed information on the responses of a tyramine receptor from *Bombyx mori* to 2-phenylethylamines and 5-phenyloxazoles (Ozoe et al., 2005, this issue) and of an octopamine receptor from *Periplaneta americana* to plant essential oils (Enan, 2005, this issue). These findings are important with respect to the limited knowledge of the pharmacological properties of tyramine and octopamine receptors, which have both been discussed as potential insecticide targets (Roeder et al., 2003).

In contrast to biogenic-amine-activated signaling, relatively little is known about the mechanism(s) involved to terminate the activity of biogenic amines. Inactivation may occur by: (1) re-uptake of the amine into the releasing cell by amine-specific transporters or (2) degradation by enzymes and/or biochemical conjugation. Recently, a family of Na^+/Cl^--dependent transport proteins with functional resemblance to mammalian transporters has been described in insects (for a review, see Caveney and Donly, 2002). This family includes a transporter that has been cloned from the cabbage looper *Trichoplysia ni* and that has high affinity for octopamine and tyramine (OAT, Malutan et al., 2002; Gallant et al., 2003). OAT-type transporters are expressed in a variety of insect orders but, surprisingly, seem to be absent in dipteran (*Drosophila melanogaster, Anopheles gambiae*) and hymenopteran (*Apis mellifera, Bombyx impatiens, Tremex columba*) species (Donly and Caveney, 2005, this issue). This indicates that the "model insects" are atypical in lacking an OAT-type transporter and probably use alternate ways to recycle these amines (Donly and Caveney, 2005, this issue). Compared with mammals, enzymes metabolizing biogenic amines, i.e., monoamine oxidase (MAO) and catechol-*O*-methyl-transferase (COMT), play a minor role, if any, in the insect nervous system (for a recent review, see Sloley, 2004). Here, the inactivation of biogenic amines is achieved by *N*-acetylation, *N*-methylation, and/or *O*-sulphation (for reviews, see Wright, 1987; Downer, 1990; Roeder et al., 2003). In addition, the β-alanyl conjugation of biogenic amines by Ebony proteins has been suggested to be involved in the inactivation of biogenic amines in *Drosophila* (Hovemann et al., 1998; Richardt et al., 2003). An ortholog of the *Drosophila ebony*-gene has now been characterized from a "larger" insect, namely the cockroach *Periplaneta americana* (Blenau and Baumann, 2005, this issue).

This short overview is meant to give an impression of the information that the reader will find in the following series of topical papers. I should like to use this opportunity to express my special thanks to the Editor-in-Chief, Dr. David Stanley, and to all our colleagues who have made this joint work possible. I am especially grateful to all authors contributing their excellent work to the present issue and the many anonymous reviewers whose comments have helped in the accomplishment of this project.

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Das dopaminerge System der Honigbiene

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Neurons with dopamine-like immunoreactivity target mushroom body Kenyon cell somata in the brain of some hymenopteran insects

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Abstract

The mushroom bodies of the insect brain are centers for olfactory and multimodal information processing and they are involved in associative olfactory learning. They are comprised of numerous (340,000 in the bee brain), small (3–8 μm soma diameter) local interneurons, the Kenyon cells. In the brain of honeybees (*Apis mellifera*) of all castes (worker bees, drones and queens), wasps (*Vespula germanica*) and hornets (*Vespa crabro*) immunostaining revealed fibers with dopamine-like immunoreactivity projecting from the pedunculus and the lip neuropil of the mushroom bodies into the Kenyon cell perikaryal layer. These fibers terminate with numerous varicosities, mainly around the border between medial and lateral Kenyon cell soma groups. Visualization of immunostained terminals in the transmission electron microscope showed that they directly contact the somata of the Kenyon cells and contain presynaptic elements. The somata of the Kenyon cells are clearly non-immunoreactive. Synaptic contacts at the somata are unusual for the central nervous systems of insects and other arthropods. This finding suggests that the somata of the Kenyon cells of Hymenoptera may serve an integrative role, and not merely a supportive function.

Keywords: *Apis mellifera*; Dopamine; Honeybee; Immunocytochemistry; Insect brain; Mushroom bodies

1. Introduction

The organization of the central nervous system (CNS) of insects and other arthropods differs fundamentally from the CNS of vertebrates in that the somata of neurons form peripheral clusters outside the neuropils. Neuropils consist of synaptically interconnected neuronal processes that are connected to their respective somata by primary neurites (Carlson, 1987). Usually, the somata of arthropod central neurons do not receive synapses and are thus probably not involved in neuronal information processing. However, some exceptions of this organizational principle have been reported.

In the giant mushroom bodies of the horseshoe crab, *Limulus polyphemus* (Xiphosura), electron microscopy revealed numerous presynaptic terminals of extrinsic fibers on the somata of the intrinsic Kenyon cells (Fahrenbach, 1979). These afferent fibers (type D) are probably identical to processes with substance P-like immunoreactivity, which originate from somata in the ventral medial ganglion and project into the somata domains of the mushroom body (Chamberlain and Engbretson, 1982).

Recently, innervation of soma clusters and soma synapses have also been found in the brain of different crustacean species. In the spiny lobster, *Panulirus*

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argus (Decapoda), many fibers with dopamine-like immunoreactivity terminate among the perikarya of the projection neurons and local interneurons of the olfactory deutocerebrum (lateral and medial soma clusters, respectively) (Schmidt and Ache, 1994). The fibers with dopamine-like immunoreactivity originate from axons descending from the eyestalk ganglia via the protocerebral tracts. Electron microscopy revealed that the terminals of these fibers in the lateral soma clusters are presynaptic to somata and primary neurites. A similar innervation of the lateral soma clusters by fibers with dopamine-like immunoreactivity also occurs in several crayfish species but not in other crustaceans (Schmidt, 1997). In a striking parallel to the innervation of the *Limulus* Kenyon cell somata by substance P-immunoreactive fibers, the lateral soma clusters of the olfactory deutocerebrum are densely innervated by a second system of descending neurons also characterized by substance P-like immunoreactivity. This system, which is present in all crustacean species studied, consists of a pair of giant protocerebral neurons whose terminals synapse on glial and neuronal elements in the lateral soma clusters (Sandeman et al., 1990; Schmidt, 1997; Schmidt and Ache, 1994).

In workers of the honeybee, *Apis mellifera* (Hymenoptera), fibers with dopamine-like immunoreactivity were found to innervate the Kenyon cell soma region (Schäfer and Rehder, 1989; Schürmann et al., 1989). These fibers had already been demonstrated earlier with the glyoxylic acid histofluorescence technique (Mercer et al., 1983). Fibers with dopamine-like immunoreactivity were also demonstrated in the soma region of the lateral protocerebral rind adjacent to the lobula of the honeybee (Schürmann et al., 1989).

In dipteran flies, varicose processes with serotonin-like immunoreactivity project into a layer distal to the lamina neuropil (Nässel, 1988; Nässel et al., 1985; Vallés and White, 1988). These processes terminate below the basal lamina in a region only occupied by photoreceptor axons and the somata of lamina monopolar cells. In the fly *Calliphora erythrocephala* (Diptera), electron microscopy showed that the serotonin-immunoreactive varicosities are enwrapped in glial processes and do not synapse onto other neuronal elements (Nässel, 1988; Nässel et al., 1985; Nässel and Elekes, 1984). From this finding the authors conclude that serotonin may be released non-synaptically from the varicosities.

The aim of the present study was to elucidate the occurrence of dopamine-immunoreactive fibers innervating the Kenyon cell soma region in the different sexes and castes of *Apis mellifera* and in other insect species. Brains of *Gryllus campestris* (Ensifera), *Schistocerca gregaria* (Cealifera), *Vespa crabro* (Hymenoptera), *Vespula germanica* (Hymenoptera), all castes of *Apis mellifera* (Hymenoptera) and *Calliphora erythrocephala* (Diptera) were studied immunocytochemically. Among the species studied we only found innervation of the Kenyon cell soma region by dopamine-immunoreactive fibers in the Hymenoptera including all three castes of *Apis mellifera*. In workers of *Apis mellifera*, presumptive synapses between fibers with dopamine-like immunoreactivity and somata of Kenyon cells were demonstrated by immuno electron microscopy.

2. Materials and methods

2.1. Animals

For the immunocytochemical procedures we used honeybee (*Apis mellifera*) worker bees, drones and queens, wasp (*Vespula germanica*) workers and hornet (*Vespa crabro*) workers, and crickets (*Gryllus campestris*), locusts (*Schistocerca gregaria*) and blowflies (*Calliphora erythrocephala*). Of all six species that were studied, at least five brains were stained with the dopamine antibody; in *Apis mellifera*, significantly more brains were used: 22 brains of worker bees, 14 brains of drones and one brain of a queen.

2.2. Immunocytochemistry; light microscopy

Insect brains were dissected under ice-cold ringer solution (270 mM NaCl, 3.2 mM KCl, 1.2 mM $CaCl_2$, 10 mM $MgCl_2$, 10 mM morpholinopropane sulfonic acid) and fixed with 2.5% or 5% freshly prepared glutaraldehyde in 0.1 M Sörensen phosphate buffer (SPB) for 2–2.5 h at room temperature (RT). Brains were washed in SPB for 4×15 min, embedded in gelatin (15% in SPB, Sigma Type A, 60 bloom) and cut into 50 μm thick serial sections using a 'Vibratome'. Mildly agitated, free-floating sections at RT were subsequently exposed to the following steps: (i) Degelatinization in warm SPB for 2×10 min; (ii) rinsing in SPB for 2×15 min; (iii) incubation in 1% sodium borohydride in SPB for 20 min; (iv) rinsing 6×5 min in SPB; (v) incubation for 2 h in a blocking solution (which was also used as antibody diluent = AD) containing 5% goat serum and 3% bovine serum albumin (BSA) in SPB with 0.3% Triton X-100 (TSPB); (vi) overnight incubation in primary antiserum — a polyclonal rabbit antibody directed against dopamine (Pel-Freez) in a dilution of 1:1000 in AD; (vii) rinsing in TSPB for 4×30 min; (viii) incubation for 4 h in CY3-labeled goat-anti-rabbit IgG (Jackson Immunoresearch) diluted 1:200 in AD; (ix) rinsing in SPB for 4×15 min; (x) mounting on slides with glycerol/SPB (1:1 mixture). The stained sections were viewed and photographed in a microscope with epifluorescence (Olympus BH-2).

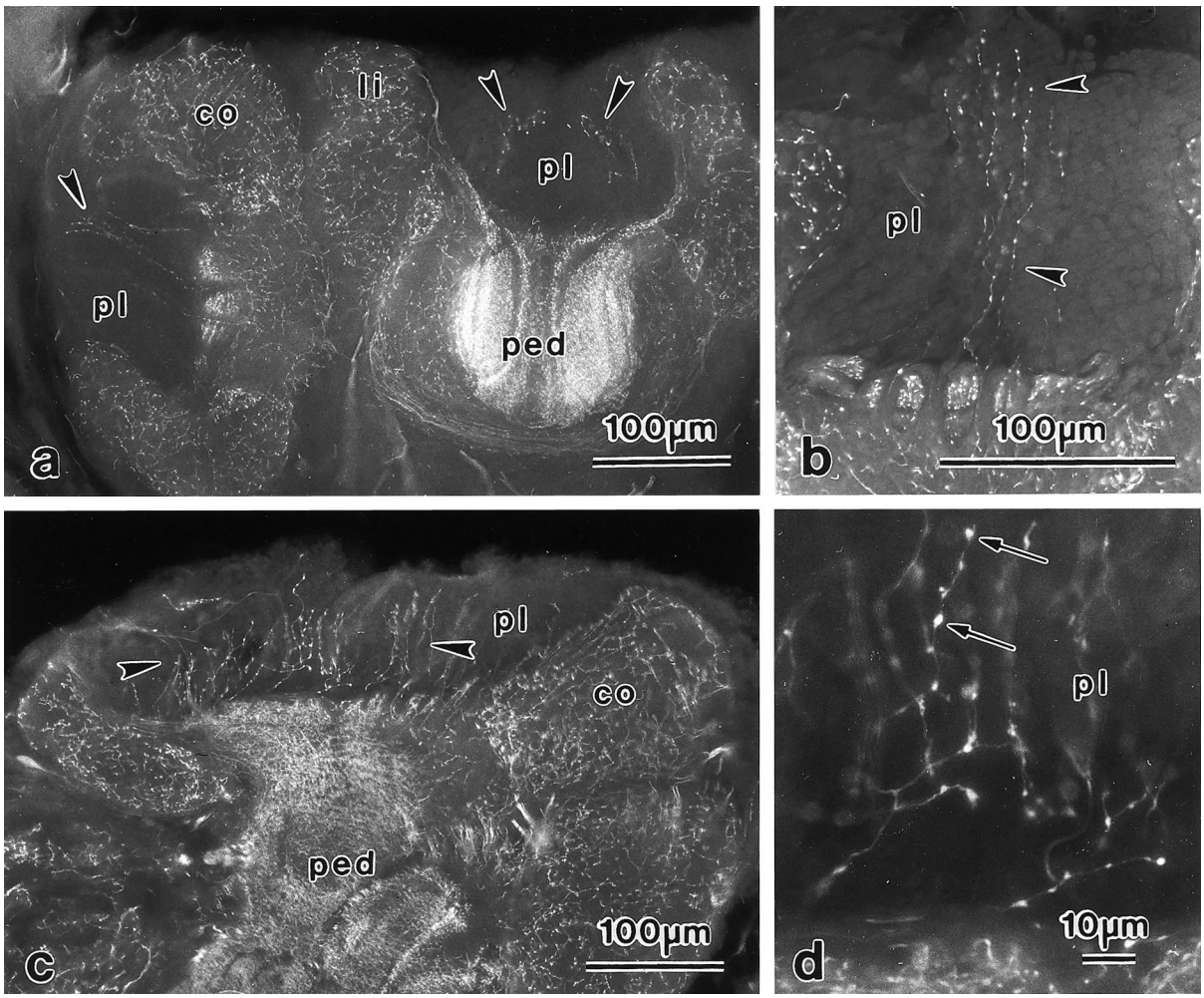

Fig. 1. Dopamine-like immunoreactivity in the mushroom bodies of the honeybee, *Apis mellifera*. LM micrographs of frontal 'Vibratome' sections. The immunostained fibers in the calycal collar (co) and lip (li) as well as in the peduncle (ped) are of extrinsic origin. Neither somata of the mushroom body perikaryal layer (pl) nor arborizations of intrinsic mushroom body cells are labeled. (a) Overview of the pattern of immunoreactivity in the mushroom body calyces and Kenyon cell perikaryal layer of a worker bee. From the pedunculus and calycal lip fibers with dopamine-like immunoreactivity (arrowheads) enter the perikaryal layer (pl). (b) Varicose fibers with dopamine-like immunoreactivity (arrowheads) in the perikaryal layer (pl) of unstained Kenyon cell somata of a worker bee median calyx. (c) Overview of the pattern of immunoreactivity in the mushroom body calyces and Kenyon cell perikaryal layer (pl) of a drone. Fibers with dopamine-like immunoreactivity (arrowheads) project from the pedunculus to the perikaryal layer (pl). (d) Arborizations of fibers with dopamine-like immunoreactivity in the perikaryal layer (pl) of a drone lateral calyx. Thick varicosities (arrows) occur at relatively regular intervals on extremely thin fibers.

Omission of the secondary antibody resulted in sections lacking specific labeling. The specificity of the primary antibody was tested by pre-adsorption in a previous study (Schmidt and Ache, 1994). According to these pre-adsorption controls, the dopamine antibody cross-reacts to $\approx 10\%$ with norepinephrine.

2.3. Immunocytochemistry; electron microscopy

A pre-embedding protocol was used to obtain immunostained ultrathin sections for electron microscopy. In short, 'Vibratome' sections of brains of *Apis mellifera* workers (fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer containing 1% sodiummetabisulfite for 3–5 h at 4°C) were incubated in anti-dopamine antibody diluted 1:1000 in TSPB containing 0.25% BSA for 24 h at 10°C. After rinsing with TSPB, sections were incubated in goat-anti-rabbit IgG (Sigma) diluted 1:40 in TSPB-BSA for 3 h at RT. After rinsing again with TSPB the sections were then incubated overnight at 10°C in a peroxidase-antiperoxidase (PAP)-complex (Boehringer) diluted 1:100 in TSPB-BSA. The final histochemical reaction was performed with 0.05% 3,3′-diaminobenzidine (DAB) diluted in 0.05 M Tris-HCl buffer and 0.01% H_2O_2 under microscopical control. After a final rinse in Tris-HCl, the sections were postfixed with 1% OsO_4 in 0.1 M cacodylate buffer for 30 min at RT, block-stained with uranyl acetate (30 min in 70% ethanol), dehydrated in ethanol, and flat-embedded on slides in

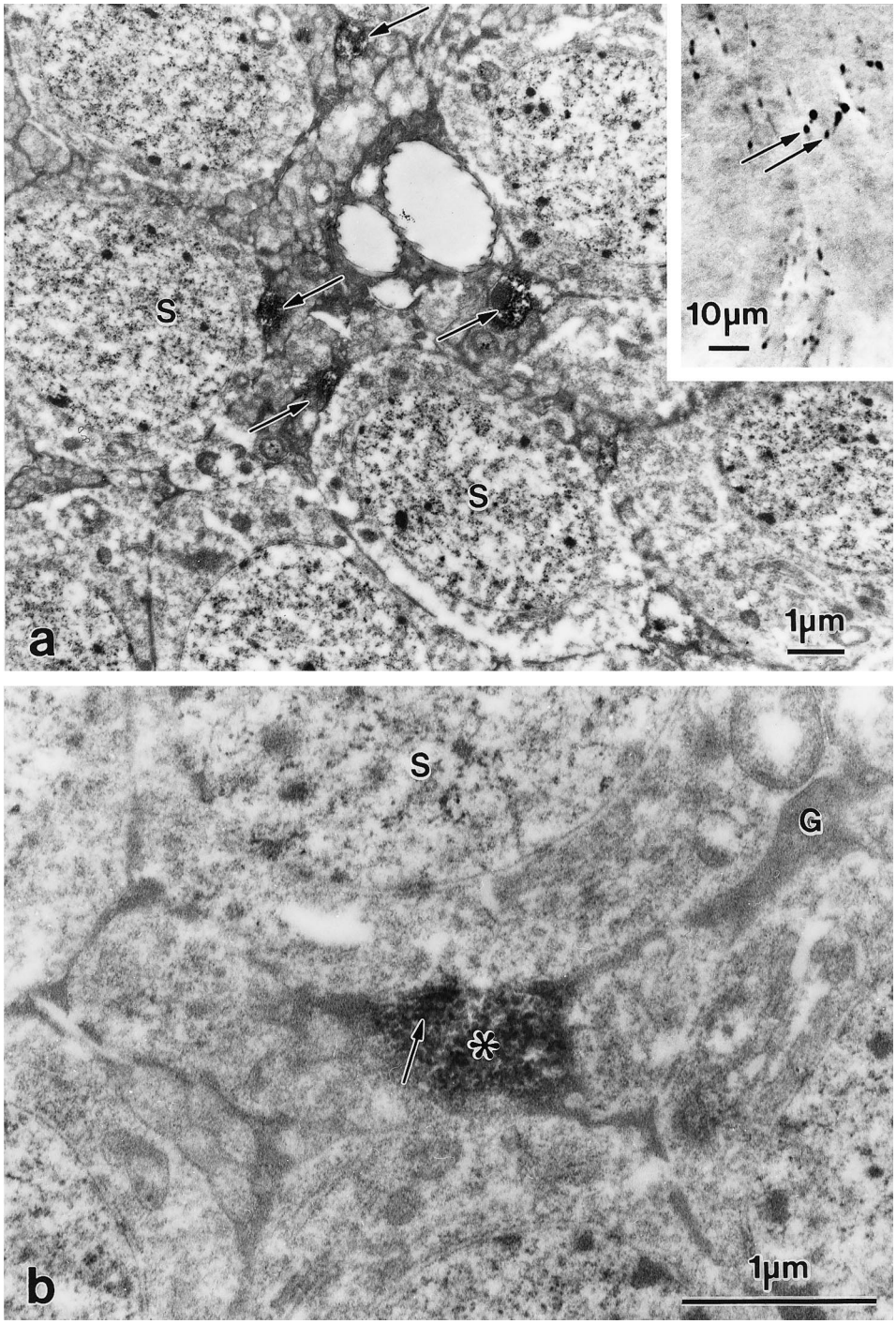

Fig. 2. Electron microscopical analysis of fibers with dopamine-like immunoreactivity in the Kenyon cell soma region of the mushroom body of the honeybee *Apis mellifera*. TEM micrographs of ultrathin sections immunostained in a preembedding (PAP/DAB) procedure. (a) Overview of neuroanatomical arrangement. Four immunostained profiles (arrows) are in direct membrane contact with Kenyon cell somata (S) or other neuronal elements. Inset: LM micrograph of a PAP/DAB immunostained 'Vibratome' section as used for electron microscopy. Arrows indicate immunoreactive boutons. (b) Contact between immunoreactive profile (asterisk) and Kenyon cell soma (S) at higher magnification. Note that membranes of the immunoreactive profile and the soma are in direct contact, whereas other neuronal elements are separated from the somata by processes of glial cells (G). Presumptive synapse characterized by pre- and postsynaptic density and an accumulation of vesicles (arrow).

araldite (Durcupan, Fluka). Ultrathin sections were cut from those areas of the sections which had been identified as containing immunostained profiles in a previous light microscopical inspection. Both, ultrathin sections contrasted with lead citrate and uncontrasted sections were viewed and photographed in the transmission electron microscope (Zeiss EM10).

3. Results

Our results demonstrate a rich innervation of the mushroom bodies of *Apis mellifera* worker bees by fibers with dopamine-like immunoreactivity of extrinsic origin, which confirms previous findings (Schäfer and Rehder, 1989; Schürmann and Elekes, 1987; Schürmann et al., 1989). The somata of intrinsic mushroom body neurons (Kenyon cells) are clearly non-labeled, and we did not detect any arborizations of Kenyon cells with dopamine-like immunoreactivity. The very dense network of fibers with dopamine-like immunoreactivity in the pedunculus and in the α- and β-lobes as well as the looser innervation of the calyces originate from a fiber bundle projecting from a small group of somata with dopamine-like immunoreactivity located below the lateral calyx of the mushroom bodies (cluster C_3 according to Schäfer and Rehder, 1989). Within the mushroom body calyces, the fibers with dopamine-like immunoreactivity are distributed unevenly and have a varicose appearance typical of aminergic neurons (Fig. 1a). The highest density of labeled fibers occurs in the collar and lip regions. A less dense innervation by immunoreactive fibers is present in the basal ring neuropil.

In the mushroom bodies of *Apis mellifera* worker bees, the dopamine antibody also labeled many fibers

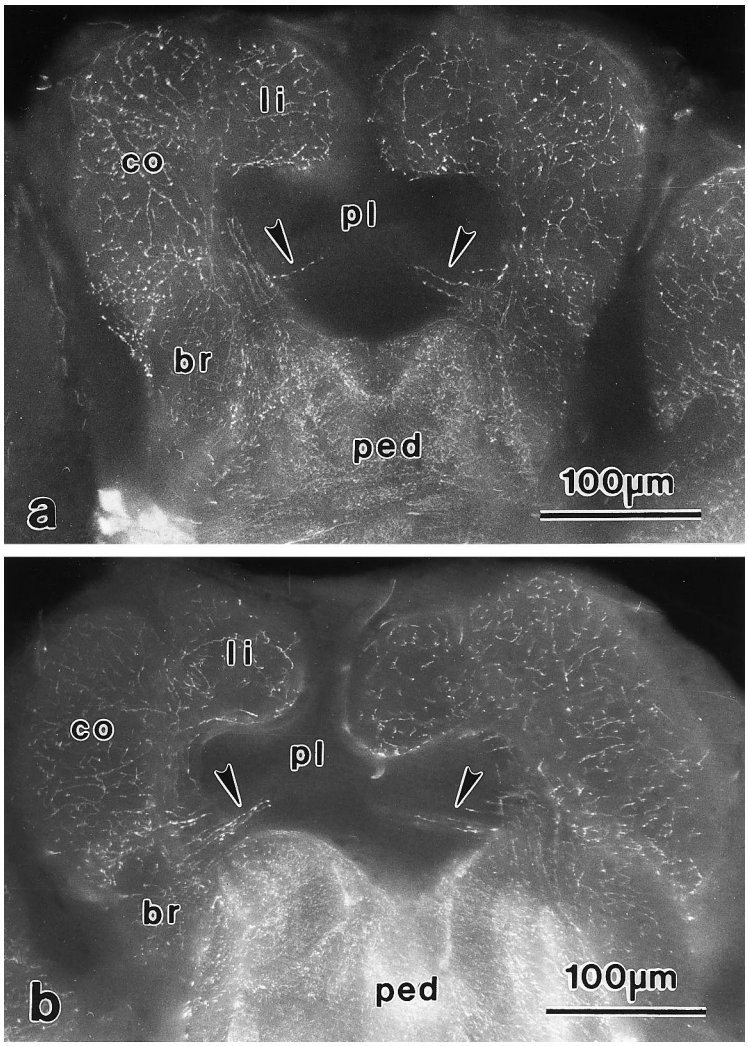

Fig. 3. Fibers with dopamine-like immunoreactivity innervating the mushroom body perikaryal layer of Hymenoptera. LM micrographs of frontal 'Vibratome' sections. Lip (li), collar (co) and basal ring (br) of mushroom body calycal neuropil as well as the stalk or pedunculus (ped) are indicated. Immunoreactive fibers (arrow heads) in the perikaryal layer (pl) are characterized by numerous varicosities. (a) *Vespula germanica*, lateral calyx. (b) *Vespa crabro*, lateral calyx.

innervating the soma region of the intrinsic mushroom body neurons, the Kenyon cells (Fig. 1a, b). The stained fibers are mainly localized around the border between medial and lateral soma groups (in the population of collar neurons) and project from the lip as well as from the peduncle neuropil into the Kenyon cell soma region (compare Schäfer and Rehder, 1989; Schürmann et al., 1989). The soma regions of the median and the lateral calyces show the same density of innervation by fibers with dopamine-like immunoreactivity (Fig. 1a). Once inside the soma cluster, the fibers with dopamine-like immunoreactivity take a rather straight course without much further branching. The fibers are very thin (diameter below 0.5 μm) over their entire length and they bear numerous bouton-like swellings (diameter up to 2 μm) that tend to be spaced very regularly (distance about 5 μm) (Fig. 1a, b). The electron microscopical analysis of this area revealed that immunostained boutons are in direct contact to the membrane of Kenyon cell somata, whereas other non-immunoreactive profiles (presumptive primary neurites) are separated from the somata by processes of glial cells (Fig. 2a, b). In some cases synapse-like structures characterized by pre- and postsynaptic densities and an accumulation of vesicles at the presynaptic face were detected in the contact zones between immunoreactive boutons and Kenyon cell somata (Fig. 2b). In all the cases the boutons with dopamine-like immunoreactivity were presynaptic to the Kenyon cell somata.

We did not detect any conspicuous differences in the innervation pattern of the Kenyon cell soma region in drones (Fig. 1c, d) and queens (data not shown) of *Apis mellifera* compared to workers, indicating that the innervation of the Kenyon cell somata is neither sex- nor caste-specific.

In all the other insect species included in this study, intense and specific dopamine-like immunoreactivity was present in many neurons of the central brain. However, only in the two other species of Hymenoptera but not in the species belonging to other insect orders, fibers with dopamine-like immunoreactivity were found to innervate the Kenyon cell soma region. In *Vespa crabro* and *Vespula germanica* (Fig. 3a, b), the innervation of the Kenyon cell soma region by fibers with dopamine-like immunoreactivity is similar to that in *Apis mellifera*, in terms of the neuroanatomical arrangement and the fiber morphology. However, in both species markedly fewer fibers than in *Apis mellifera* project into the Kenyon cell soma region, and these fibers do not extend as far into the soma cluster.

In insect species not belonging to the order Hymenoptera (i.e. *Gryllus campestris, Schistocerca gregaria* and *Calliphora erythrocephala*) we could not detect fibers with dopamine-like immunoreactivity projecting into the Kenyon cell soma region (data not shown). This is in agreement with the results of other studies mapping the distribution of dopamine-like immunoreactivity in the brain of the cockroach *Periplaneta americana* (Milton et al., 1991), the locust *Schistocerca gregaria* (Wendt and Homberg, 1992) and the flies *Calliphora erythrocephala, Phormia terraenovae* and *Drosophila melanogaster* (Nässel and Elekes, 1992; Nässel et al., 1988).

4. Discussion

The mushroom bodies of the insect brain are considered a prominent area for olfactory information processing and learning (for reviews see Davis, 1993; Menzel et al., 1994) but only little is known about possible neurotransmitters of the intrinsic Kenyon cells. The majority of intrinsic Kenyon cells react with antibodies raised against taurine (Bicker, 1992; Schäfer et al., 1988). Certain populations of intrinsic cells in the bee are immunoreactive with antisera against neuropeptides, in particular FMRFamide (Schürmann and Erber, 1990) and gastrin/CCK (Kloppenburg et al., 1990). Faint glutamate-like immunoreactivity has been described in subpopulations of Kenyon cells in the bee (Bicker et al., 1988), and in the cricket (Schürmann et al., 1998). However, most neuroactive compounds have so far only been demonstrated in extrinsic mushroom body neurons, which connect the mushroom bodies with other brain neuropils. For example, various parts of the mushroom bodies of the honeybee are innervated by varicose processes from a few amine-containing neurons (Kreissl et al., 1994; Schäfer and Rehder, 1989; Schürmann et al., 1989; Schürmann and Klemm, 1984). Our study extends the initial finding of Schäfer and Rehder (1989) and Schürmann et al. (1989) that the somata of Kenyon cells in the worker honeybee are innervated by fibers with dopamine-like immunoreactivity. Data presented in this study allow the generalization that in all castes of *Apis mellifera* and in additional different species of Hymenoptera the Kenyon cell soma region is targeted by prominent extrinsic innervation by neurons with dopamine-like immunoreactivity. We further provide the first direct evidence that in workers of *Apis mellifera* the fibers with dopamine-like immunoreactivity are presynaptic to the Kenyon cell somata.

The polarity of this synaptic connection indicates that the direction of information flow is from the fibers with dopamine-like immunoreactivity to the Kenyon cell somata. This strongly indicates a modulatory type of interaction mediated by dopamine, which is consistent with the general concept of the functional roles of biogenic amines in the CNS of arthropods (Bicker and Menzel, 1989; Erber et al., 1993; Evans, 1980). The

putative functions of this presumptive modulatory interaction at the somata of neurons, which is very unusual in the CNS of arthropods, can be inferred from comparisons with other nervous systems. The rather similar innervation of the lateral soma clusters in the brain of the spiny lobster (*Panulirus argus*) by descending neurons with dopamine-like immunoreactivity (Schmidt and Ache, 1994) is especially interesting. In that system it was shown that the olfactory projection neurons, whose somata constitute the lateral clusters, are dye and electrically coupled in the vicinity of the soma/primary neurite (Wachowiak and Ache, 1994). Electron microscopy revealed contact zones characterized by large gap junction-like membrane specializations between somata and neurites of other olfactory projection neurons in the lateral soma clusters (Schmidt and Ache, 1994). Since these contact zones between somata and neurites only occur in conjunction with a presumptive dopamine fiber which is in direct membrane contact with both of them and presynaptic to either one of them, Schmidt and Ache (1994) proposed that the extent of electrical coupling between olfactory projection neurons could be modulated by the activity of descending dopaminergic neurons. It is possible that the fibers with dopamine-like immunoreactivity innervating the Kenyon cell somata of Hymenoptera serve a similar function. In another species of Hymenoptera, the wood ant *Formica lugubris*, it has been shown that the glial processes ensheathing the Kenyon cell somata are not continuous but fenestrated by areas of direct soma-somatic or rarely soma-axonic contacts between neurons (Landolt and Ris, 1966). Structurally, these junctions resemble gap junctions, mediating electrical coupling in other species (Landolt and Ris, 1966). In insects, electrical coupling of Kenyon cells has so far not been shown directly. However, measurements of evoked potentials (Kaulen et al., 1984; Mercer and Erber, 1983) and the synchronous activity of Kenyon cells observed after electrical stimulation of the antennal nerve (Maynard, 1967) or after the presentation of odors (Laurent and Naraghi, 1994; Stopfer et al., 1997) can be interpreted as indirect evidence. Targeting the presumptive modulatory input of dopaminergic fibers to the somata and/or primary neurites of the intrinsic mushroom body neurons may be an effective way to assure an even distribution of the induced changes over the entire arbor of a given neuron. The accessibility of honeybee Kenyon cell somata to patch-clamp recordings in vitro (Schäfer et al., 1994) makes them an ideal system for investigating the functional modulatory role of dopamine in information processing and the cellular mechanisms involved.

A prerequisite of dopamine acting on the Kenyon cell somata is the existence of specific dopamine receptors on these somata. A dopamine receptor specifically expressed in the mushroom bodies (DAMB) was cloned from *Drosophila* (Han et al., 1996). Although DAMB transcripts were preferentially expressed in the Kenyon cell somata, immunohistochemical analysis revealed that DAMB receptors were highly enriched in the axonal elements (pedunculi, α-, β- and γ-lobes) of the mushroom bodies (Han et al., 1996). These observations suggest that the DAMB receptor primarily functions in the axons of mushroom body cells. Recently, the honeybee homologue of this D1-like receptor (*Am*BAR6) was cloned (Ebert et al., 1998; Kokay et al., 1999). In situ-hybridization revealed that the expression of the *Am*BAR6 gene is restricted to the Kenyon cell somata within and around the calyces of the mushroom bodies (Humphries and Ebert, 1998; Kokay et al., 1999). Blenau et al. (1998) have isolated another dopamine receptor cDNA, *Am*Dop1, from the honeybee. In situ-hybridization to tissue sections of adult honeybee brain revealed that the *Am*Dop1 receptor gene is expressed in neurons of different brain neuropils, including mushroom body intrinsic neurons (Blenau et al., 1998). Using antibodies directed against different regions of the *Am*DOP1 receptor polypeptide, specific staining of mushroom body soma as well as neuropil regions was found (Blenau and Baumann, personal observation). This makes this AmDOP1 receptor one possible candidate for mediating the actions of dopamine in the Kenyon cell soma region.

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Research report

Analysis of two D1-like dopamine receptors from the honey bee *Apis mellifera* reveals agonist-independent activity

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Abstract

Dopamine is found in many invertebrate organisms, including insects, however, the mechanisms through which this amine operates remain unclear. We have expressed two dopamine receptors cloned from honey bee (*Am*DOP1 and *Am*DOP2) in insect cells (*Spodoptera frugiperda*), and compared their pharmacology directly using production of cAMP as a functional assay. In each assay, *Am*DOP1 receptors required lower concentrations of dopamine and 6,7-ADTN for maximal activation than *Am*DOP2 receptors. Conversely, butaclamol and *cis*(*Z*)-flupentixol were more potent at blocking the cAMP response mediated through *Am*DOP2 than *Am*DOP1 receptors. Expression of *Am*DOP1, but not *Am*DOP2, receptors significantly increased levels of cAMP even in the absence of ligand. This constitutive activity was blocked by *cis*(*Z*)-flupentixol. This work provides the first evidence of a constitutively activated dopamine receptor in invertebrates and suggests that although *Am*DOP1 and *Am*DOP2 share much less homology than their vertebrate counterparts, they display a number of functional parallels with the mammalian D1-like dopamine receptors.

Theme: Neurotransmitters, modulators, transporters, and receptors

Topic: Catecholamine receptors

Keywords: G protein-coupled receptor; Biogenic amine; Invertebrate; cAMP; Baculovirus

1. Introduction

Dopamine has been detected in many insect species, but its functions, and the mechanisms through which it operates in the insect brain, remain largely unresolved. In the honey bee, *Apis mellifera*, dopamine has been implicated in olfactory learning and memory [25,27–29], modulation of motor output [26] and adult development and behaviour [6,39,46,49]. To help establish the role of dopamine in these processes, it is important to characterize the receptor proteins through which dopamine acts. One problem to date, has been a lack of information about pharmacological tools that selectively alter neurotransmission in dopaminergic pathways in the insect brain, or that selectively interact with specific subtypes of insect dopamine receptor.

Insect cellular responses to dopamine, as in vertebrates, are mediated via G protein-coupled receptors (reviewed in Ref. [1]). These receptors are members of a large family of proteins that contain seven transmembrane domains and are coupled to various signal transduction pathways via trimeric G (guanine nucleotide binding) proteins. The dopamine receptors of vertebrates have been categorized into two major subfamilies, D1 receptors and D2 receptors [21]. Activation of members of the D1 receptor family, which includes D1 (D1A) and D5 (D1B) receptors, leads to an increase in intracellular cAMP levels, whereas receptors from the D2 receptor subfamily, which includes

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receptor subtypes D2, D3 and D4, either reduce cAMP levels or act via different second messenger pathways when stimulated. As well as being differentiated on the basis of coupling to the cAMP pathway, vertebrate dopamine receptors can be distinguished on the basis of their ligand binding profiles (reviewed in Refs. [30,40,48]). For example, the benzazepine, *R*(+)-SCH 23390, binds with much higher affinity to vertebrate D1 receptors than to D2 receptors, whereas benzamides, such as sulpiride and eticlopride, and the butyrophenone, spiperone, show higher affinity binding to vertebrate D2 receptors than to members of the vertebrate D1 receptor subfamily. The D1 and D2 receptor subfamilies can also be differentiated using agonists such as SKF 38393, a benzazepine that activates vertebrate D1 receptors at significantly lower concentrations than D2 receptors.

Studies characterizing dopamine receptors in brain tissue from a variety of insects, including cockroach [34,35], locust [9], sphinx moth [14] and honey bee [2,4,23], suggest that insect dopamine receptors are pharmacologically distinct from those found in vertebrates. This suggestion was supported by the cloning and in vitro characterization of two dopamine receptors from *Drosophila*, DAMB/DopR99B [12,15] and *Dmdop*1/*d*DA1 [13,45]. Both of these receptors are positively coupled to adenylyl cyclase, but neither exhibits a pharmacological profile typical of a vertebrate D1 receptor. For example, the vertebrate D1 receptor antagonist, SCH 23390, does not exhibit high affinity binding, nor is it particularly effective at blocking cAMP stimulation via these *Drosophila* dopamine receptors [13,45]. Moreover, the agonist activity of SKF 38393 at these receptors is low compared to vertebrate dopamine receptors [37,45].

The orthologs of *Dmdop*1/*d*DA1 and DAMB/DopR99B have been cloned from the honey bee, *Apis mellifera*. *Amdop1*, the ortholog of *Dmdop*1/*d*DA1, encodes a dopamine receptor (*Am*DOP1) that is not only positively coupled to adenylyl cyclase [3] (accession no. Y13429), but also, like the *Dm*DOP1/*d*DA1 receptor, exhibits a pharmacological profile that is distinct from that of vertebrate D1 receptors [3]. Most strikingly, the vertebrate D2 receptor antagonist spiperone was found to have a higher affinity for *Am*DOP1 than SCH 23390. A second honey bee dopamine receptor gene, *Amdop2*, the ortholog of DAMB/DopR99B [11,17] (previously known as *Ambar6* or *Apis mellifera* biogenic amine receptor 6; accession no. AF498306), encodes a protein which is also positively coupled to adenylyl cyclase, but as yet, little is known about the pharmacology of this receptor. In situ analysis of *Amdop1* [3] and *Amdop2* [17] transcripts revealed that these two receptors have distinct, but overlapping, patterns of expression in the brain.

*Am*DOP1 and *Am*DOP2 receptors are both like the vertebrate D1 receptor class in that their activation leads to increased levels of intracellular cAMP, but the amino acid sequences of these two honey bee dopamine receptors are much more divergent than the mammalian D1 receptors, D1/D1A and D5/D1B. The human D1/D1A and D5/D1B receptors have an amino acid sequence identity of ~80% across their transmembrane domains, whereas *Am*DOP1 and *Am*DOP2 contain only about 50% identity in these regions. Phylogenetic analysis reveals that *Am*DOP1 is more closely related to the vertebrate D1 receptors than *Am*DOP2, which groups instead with the vertebrate α_1-adrenergic receptors [17,22]. While the human D1/D1A and D5/D1B receptors share a high level of homology, functional differences between these two receptors have been identified. In vitro studies have revealed that expression of the human D5/D1B receptor results in an agonist-independent increase in intracellular cAMP, whereas similar expression levels of the human D1/D1A receptor do not elevate cAMP levels [47]. Agonist-independent (constitutive) activity has also been shown for dopamine receptors cloned from other vertebrates, and this property is now considered a distinguishing characteristic of the vertebrate D5/D1B receptor subclass [33].

Using the baculovirus *Autographa californica* nucleopolyhedrovirus as an expression vector, we have expressed *Am*DOP1 and *Am*DOP2 in insect (*Spodoptera frugiperda*) cells in order to examine in parallel the pharmacological properties and functional characteristics of these two honey bee dopamine receptors. Measurements of cAMP production are used as a functional assay to examine the agonist and antagonist activities of selected dopamine receptor ligands. The results reveal that these two receptors have similar, but distinct, pharmacological profiles, and that *Am*DOP1, like the D5/D1B class of vertebrate dopamine receptors, exhibits agonist-independent activity.

2. Materials and methods

2.1. Construction of baculovirus recombinants

A baculovirus encoding *Amdop1* under the control of the polyhedrin promoter was constructed as follows. An ~1750 bp fragment containing the coding region of the *Amdop1* gene was isolated from the pBluescript II vector [3] using a *Pst*I site in the vector just upstream of the translation start site and a *Xho*I site 520 bp downstream of the stop codon. This fragment was then ligated into the donor vector pFastBac (Invitrogen, Bac-To-Bac Baculovirus expression system) that had also been digested with *Pst*I and *Xho*I. A segment of the pFastBac construct containing the *Amdop1* coding region under the control of the polyhedrin promoter was then recombined from the donor plasmid into the baculovirus genome bacmid (bMON14272) using the Tn7 transposase in *E. coli* (strain DH10Bac). The bacmid containing the *Amdop1* coding region was then purified and transfected into Sf21 cells using CellFectin reagent (Invitrogen). A baculovirus re-

combinant expressing the *Amdop2* gene was constructed as described in Humphries et al. [17]. Briefly, a *Bgl*II site 30 bp upstream of the translational start site and an *Xba*I site 65 bp downstream of the stop codon were used to subclone the *Amdop2* coding region into pFastBac. The region containing *Amdop2* and the polyhedrin promoter was then recombined into the bacmid and transfected into Sf21 cells as described above. A control virus not expressing either receptor was made by transfecting the unaltered bMON14272 baculovirus genome bacmid into Sf21 cells. Stocks of virus were produced using protocols described in the Bac-To-Bac manual.

2.2. Expression of AmDOP1 and AmDOP2 receptors in Sf21 and Sf9 cells

Exponentially growing insect cells (either Sf21 or Sf9 cells) were diluted into serum-free medium (Sf-900 II SFM, Invitrogen). Cells (1.8×10^5/well) were aliquoted into 24-well culture dishes and allowed to adhere overnight at 28 °C before being infected with virus at a multiplicity of infection of five. All cells were then incubated at 28 °C for 28 h before being used for intracellular cAMP assays or Northern analysis.

2.3. Northern analysis

Total RNA was isolated from Sf21 cells infected by virus expressing either *Amdop1* or *Amdop2*. Total RNA isolated from cells infected with control virus (see above) was also examined. TRIzol LS reagent (Invitrogen) was used for RNA extraction as described by the manufacturer. Northern analysis was carried out as described by Sambrook et al. [38], with minor modifications. RNA (5 μg) was loaded onto a 1% agarose MOPS-formaldehyde gel and fractionated by electrophoresis. RNA molecular weight marker II (Roche) was used as a size standard. For assessment of RNA loading, the gel was stained with 5 μg/ml ethidium bromide in 1× MOPS buffer, destained in several changes of distilled water, and photographed. RNA was transferred onto positively charged nylon membranes (Roche) overnight by capillary action using 20× SSC as transfer buffer, and then fixed on to the membrane by baking at 120 °C for 30 min. Probes for *Amdop1* and *Amdop2* were labeled with ^{32}P using random primer DNA labeling (GibcoBRL) with fragments containing the entire coding regions of *Amdop1* and *Amdop2* as templates. Labeled probes were purified on Sephadex NICK columns (Pharmacia). The membranes were prehybridized in 50% formamide, 5× SSC, 5× Denhardt's, 0.1 mg/ml denatured salmon sperm DNA, and 0.1% SDS at 42 °C for 2 h. *Amdop1* or *Amdop2* (50,000 counts/ml) probe was then added, and allowed to hybridize 16 h at 42 °C. Blots were washed in 1× SSC, 0.1% SDS for 5 min at room temperature and then twice at 60 °C for 30 min. The membrane was then exposed to X-ray film for 12–72 h at −80 °C. Quantitation of Northern blots and ethidium bromide stained gels was done using NIH Image.

2.4. Analysis of cAMP levels

Measurements of intracellular cAMP levels were used to monitor the effects of amines, and selected amine-receptor agonists and antagonists, on cells expressing either *Am*DOP1 or *Am*DOP2 receptors. Uninfected cells were included as a control. After incubating cells for 28 h in serum-free medium, this medium was removed and replaced with fresh medium containing 10^{-4} M of the cAMP phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) and the selected drug(s) at concentrations indicated in the figure legends. Cells were exposed to the drug(s) for 20 min at 28 °C. The medium bathing the cells was then removed, the cells were lysed with lysis buffer 1B (supplied in the cAMP assay kit) and the amount of intracellular cAMP was determined using a cAMP enzyme immunoassay (Amersham Pharmacia Biotech). Each sample was analyzed in duplicate. A minimum of three independent assays was carried out for each compound.

2.5. Pharmacology of the AmDOP2 receptor

A pharmacological profile for *Am*DOP1 has been presented elsewhere [3]. Prior to comparing responses mediated via *Am*DOP1 and *Am*DOP2 receptors, the pharmacology of the *Am*DOP2 receptor was examined. The effects of the biogenic amines dopamine, norepinephrine, octopamine, tyramine, serotonin, and histamine on intracellular cAMP levels were investigated in Sf21 cells expressing the *Am*DOP2 receptor. Uninfected cells also were examined. For each experiment undertaken in this component of the study, measurement of the level of cAMP in the absence of ligand established the baseline of 100%, and all other measurements are expressed as a percentage of that baseline.

To examine further the pharmacological profile of *Am*DOP2, the activity of the following vertebrate dopamine receptor agonists was tested on cells expressing the *Am*DOP2 receptor: SKF 38393, 2-amino-6,7-dihydroxy-1,2,3,4-tetra-hydronaphthalene (6,7-ADTN), apomorphine, lisuride, and 6-chloro-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine (6-chloro-APB). Each ligand was added at a concentration of 10^{-5} M to serum-free medium containing 10^{-4} M IBMX. After exposing cells to ligand for 20 min, cAMP levels were measured as described above. For purposes of comparison, data are expressed as a percentage of the level of cAMP measured in the presence of 10^{-5} M dopamine.

Antagonists were examined for their ability to block dopamine-mediated activation of the *Am*DOP2 receptor. The antagonist SCH 23390 was chosen because of its known selectivity at vertebrate D1 receptors, while spiperone, eticlopride, and domperidone are selective for

vertebrate D2 receptors. To enable comparisons to be made with data from previous studies of insect dopamine receptors, chlorpromazine, fluphenazine, haloperidol, metoclopramide, *cis*(*Z*)-flupentixol, and butaclamol were also tested. In addition, the vertebrate D2 receptor agonist, lisuride, was examined for antagonistic activity after it was discovered that it did not act as an agonist at the *Am*DOP2 receptor. The selected antagonist (10^{-5} M) was combined with 10^{-5} M dopamine immediately before both were applied to cells as described above. Levels of cAMP recorded after the application of dopamine plus antagonist are expressed as a percentage of the level of cAMP recorded in cells exposed to 10^{-5} M dopamine alone.

2.6. Comparison of AmDOP1 and AmDOP2 pharmacology

To compare the pharmacology of the two honey bee dopamine receptors, the *Am*DOP2 receptor was re-examined in assays in which cells expressing the *Am*DOP1 receptor were also tested. Responses to the endogenous ligand dopamine, and to the agonist 6,7-ADTN, were examined in the two groups of cells. Agonists were applied to the cells for 20 min, as described above. In a second set of experiments, four antagonists were examined for their ability to block dopamine-mediated cAMP production via the two receptors. As preliminary experiments revealed that dopamine was more potent at activating *Am*DOP1 receptors than *Am*DOP2 receptors (see Results), 10^{-6} M dopamine was used to analyze the actions of dopamine-receptor antagonists at *Am*DOP1 receptors, rather than 10^{-5} M dopamine, the concentration used to examine the effectiveness of antagonists acting via *Am*DOP2 receptors. After exposing cells to dopamine plus the selected antagonist, cAMP levels were analysed as described above. The number of independent assays carried out for each compound is indicated in the figure legends.

2.7. Drugs

Dopamine hydrochloride; (−)-norepinephrine bitartrate; tyramine hydrochloride; DL-octopamine hydrochloride; 5-hydroxytryptamine creatinine sulfate; histamine dihydrochloride; 3-isobutyl-1-methylxanthine (IBMX); haloperidol, spiperone, chlorpromazine hydrochloride, and (−)-sulpiride were purchased from Sigma. *R*(+)-SCH 23390 hydrochloride, metoclopramide hydrochloride, fluphenazine dihydrochloride, domperidone, *cis*(*Z*)-flupentixol dihydrochloride, *R*(+)-SKF 38393 hydrochloride, *S*(−)-eticlopride hydrochloride, *R*(+)-lisuride hydrogen maleate, (+)-butaclamol hydrochloride, (−)-apomorphine hydrochloride, (±)-6-chloro-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrogen bromide (6-chloro-APB), and (±)-2-amino-6,7-dihydroxy-1,2,3,4-tetra-hydronaphthalene hydrobromide (6,7-ADTN) were obtained from Research Biochemicals International.

2.8. Data analysis

Data were analyzed and displayed using Prism 3.0a for Macintosh (GraphPad Software, San Diego, CA, USA, www.graphpad.com). Curve fitting was done using least-squares analysis. Statistical significance was determined using one-way analysis of variance (ANOVA) with *P* values <0.05 considered significant. Where appropriate, Tukey–Kramer multiple comparison tests were used for post hoc analysis of differences between groups.

3. Results

3.1. Analysis of the activity of biogenic amines at the AmDOP2 receptor

Fig. 1 shows the responses to a range of biogenic amines

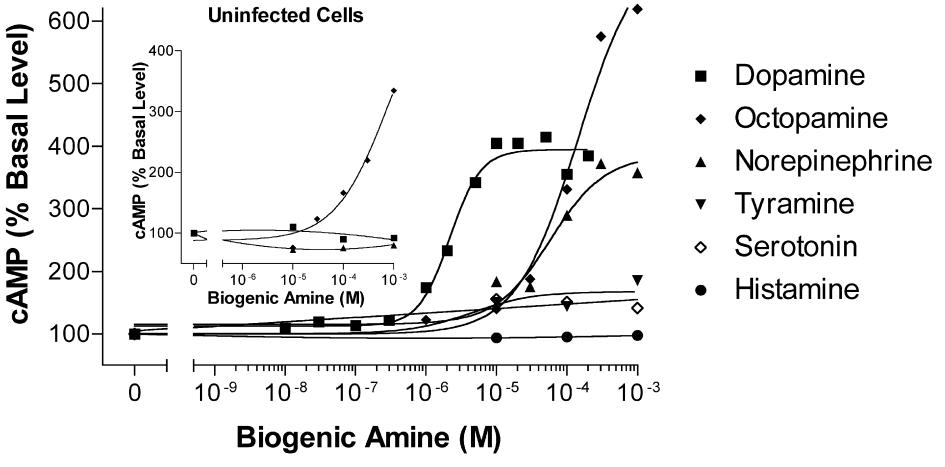

Fig. 1. Biogenic amine-mediated changes in intracellular cAMP level. Responses were examined in Sf21 cells expressing the *Am*DOP2 receptor, as well as in control (uninfected) cells (inset). All measurements are expressed relative to basal (cAMP detected in the absence of ligand), which provides the baseline of 100%. Curves shown are typical results, and are representative of three to six independent experiments done in duplicate.

of Sf21 cells expressing the *Am*DOP2 receptor. Of the biogenic amines tested, dopamine was the most potent (Fig. 1, EC_{50} 2.2 μM), and increased cAMP levels approximately fourfold above basal levels. Stimulation of the *Am*DOP2 receptor with norepinephrine also increased cAMP levels approximately fourfold above the basal level, but the EC_{50} for this amine was more than 20-fold higher than that observed for dopamine (Fig. 1, EC_{50} 58 μM). Treatment with high concentrations ($>10^{-5}$ M) of octopamine also elicited a response. However, even at the highest concentration tested, 10^{-3} M, the octopamine response did not reach a plateau which prevented us from determining an EC_{50} value for this amine. Small increases in cAMP levels could be induced also by treatment of cells with high concentrations of tyramine and serotonin, whereas histamine had no observable effect on cAMP levels. To examine the possibility that dopamine, octopamine or norepinephrine may be acting via a receptor, or receptors, endogenous to Sf21 cells (see Ref. [36]), uninfected (control) cells were treated with these amines (Fig. 1, inset). Analysis of uninfected cells suggested that responses to octopamine (Fig. 1) are mediated, at least in part, by an endogenous octopamine receptor, as high concentrations of octopamine ($>10^{-5}$ M) increased cAMP levels in these cells (Fig. 1 inset). However, neither dopamine nor norepinephrine, even at very high concentrations, altered cAMP levels in uninfected cells, indicating that the effects of these amines on cAMP levels in cells expressing the *Amdop2* gene are mediated via *Am*DOP2 receptors.

3.2. Identification of synthetic agonists and antagonists of the AmDOP2 receptor

At a concentration of 10^{-5} M, the vertebrate dopamine receptor agonists, 6,7-ADTN, 6-chloro-APB and apomorphine stimulated cAMP production to a level similar to that produced by 10^{-5} M dopamine (Fig. 2A). However, treatment with lisuride, a potent D2 receptor agonist, or the vertebrate D1 receptor agonist SKF 38393, did not increase cAMP significantly above basal levels; indeed, lisuride was found to act as an antagonist rather than an agonist at the *Am*DOP2 receptor (see Fig. 2B).

Among the antagonists tested, chlorpromazine, *cis*(*Z*)-flupentixol, fluphenazine, butaclamol, SCH 23390, haloperidol, and spiperone, all reduced dopamine-mediated stimulation of cAMP to a level not significantly different from basal levels recorded in the absence of dopamine (Fig. 2B). Significantly less effective at blocking dopamine activation of the *Am*DOP2 receptor were the antagonists metoclopramide, sulpiride, and eticlopride. Together with published data on the pharmacology of the *Am*DOP1 receptor [3], these 'point' assays examining the pharmacology of *Am*DOP2 enabled us to decide which ligands to use to compare directly the pharmacology of these two honey bee dopamine receptors.

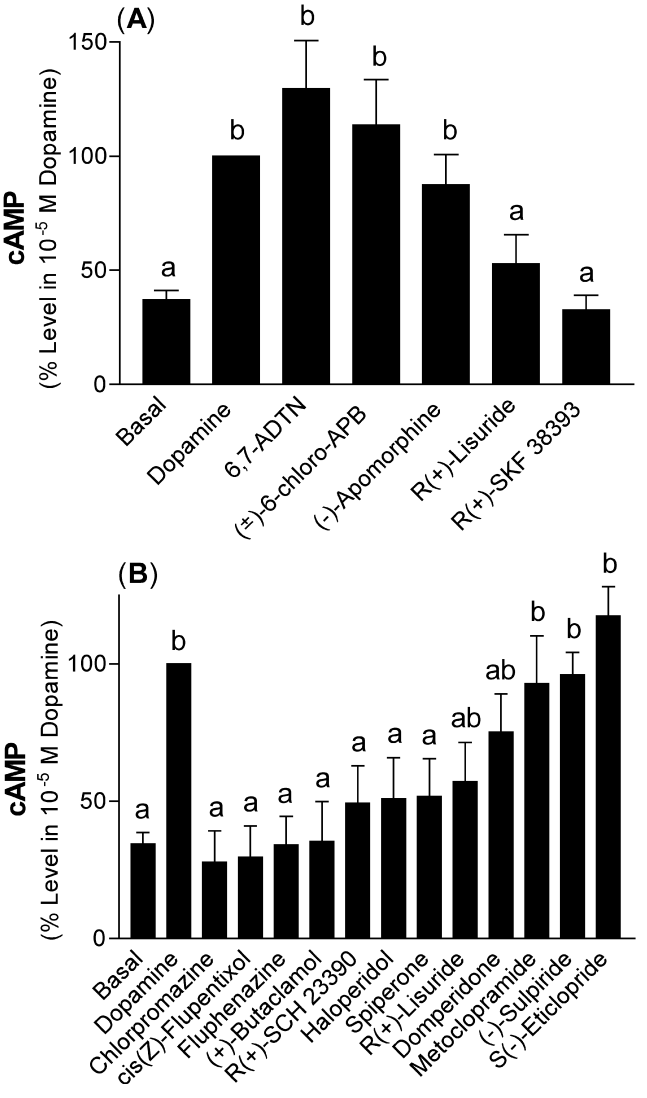

Fig. 2. Responses of Sf21 cells expressing the *Am*DOP2 receptor to (A) selected dopamine receptor agonists at a concentration of 10^{-5} M, and (B) dopamine (10^{-5} M) plus 10^{-5} M of one of a selection of amine receptor antagonists. Values are expressed as a percentage of the cAMP level in 10^{-5} M dopamine, and are the means±S.E.M. of three or four independent experiments. Basal levels of cAMP (Basal) were recorded in the presence of IBMX alone. Overall statistical significance was determined by one-way ANOVA followed by Tukey–Kramer tests. For the agonists (A), $F=15.15$ and $P<0.0001$. For the antagonists (B), $F=8.99$ and $P<0.0001$. Letters over each bar on the graph indicate whether or not differences between groups are significant. Groups that share a letter are not significantly different ($P>0.05$).

3.3. Comparison of AmDOP1 and AmDOP2 receptor pharmacology

The *Am*DOP1 and *Am*DOP2 receptors were assessed under identical conditions to allow direct comparison of pharmacological responses. Northern analysis was used to examine the specificity of expression of the two dopamine receptor genes (Fig. 3). RNA isolated from cells expressing *Am*DOP1 receptors, and cells expressing *Am*DOP2 receptors, was hybridized with probe for *Amdop1* (Fig.

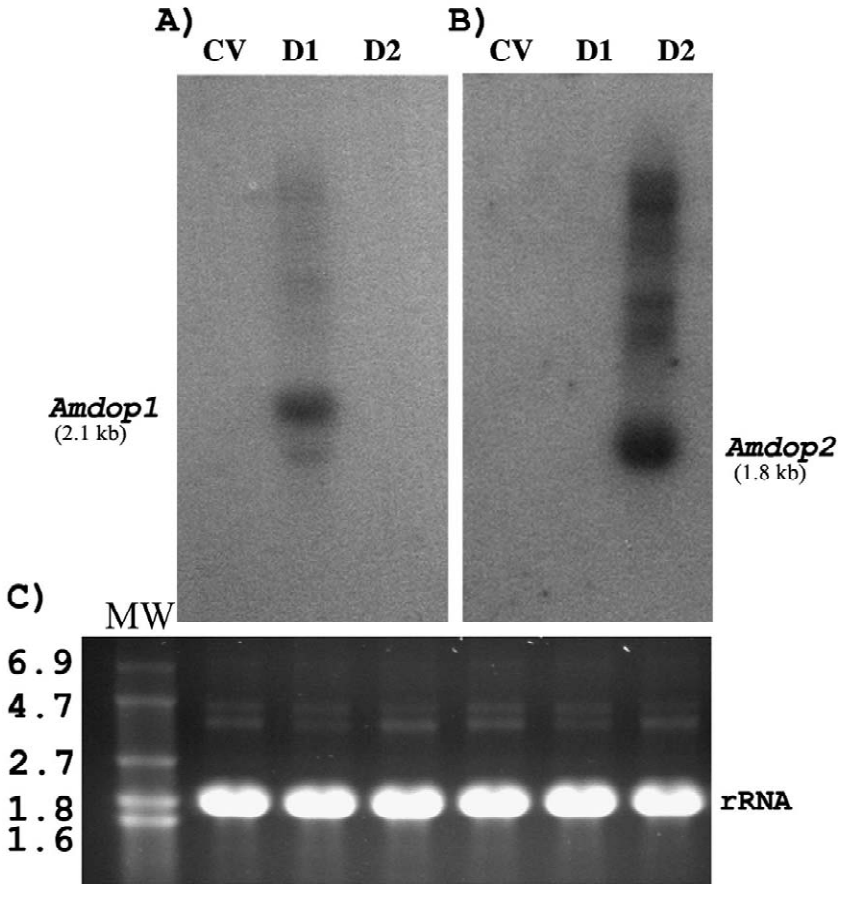

Fig. 3. Northern analysis of *Amdop2* and *Amdop1* expression levels. Total RNA was isolated from Sf21 cells infected with baculovirus expressing *Amdop2* (D2), *Amdop1* (D1), or no receptor (control virus, CV). (A) Membrane probed with *Amdop1* shows a specific band at 2.1 kb, the expected size for the *Amdop1* transcript produced by the baculovirus construct. (B) Membrane incubated with *Amdop2* probe reveals a band at 1.8 kb, the expected size for the *Amdop2* transcript produced by the recombinant baculovirus. (C) Ethidium bromide staining of the gel before transfer shows equivalent loading of total cellular RNA. The sizes of the molecular weight markers are indicated in kilobases. The most intense band (rRNA) contains the 18S rRNA and the 28S rRNA that dissociates into two equally sized subunits due to the 'hidden break' in insect 28S rRNA [18].

3A), and for *Amdop2* (Fig. 3B). RNA isolated from cells infected with control virus (see Methods) was included as a control. The loading of RNA on the gel is shown with ethidium bromide staining in Fig. 3C. The 28S rRNA of many insect species dissociates into two equally sized subunits under denaturing conditions [18], and these subunits are not resolved from the 18S rRNA. After Northern hybridization, specific bands corresponding to the expected construct sizes were observed. Quantitation of the *Amdop1* and *Amdop2* signals with respect to the rRNA band, revealed that *Amdop1* was expressed at about 63% of the level of *Amdop2* expression. A number of higher molecular weight bands were also recognized by the *Amdop1* and *Amdop2* probes, and these minor bands may be due to the production of 'run on' transcripts from the baculovirus constructs.

Elevated levels of cAMP were observed in cells infected by virus expressing *Am*DOP1. In 13 independent assays in which Sf21 cells expressing *Am*DOP1 receptors, cells expressing *Am*DOP2 receptors, and control (uninfected) cells were examined in parallel, cAMP levels were, on average, more than fourfold higher (4.6±0.8 S.E.M.) in cells expressing *Am*DOP1 than in uninfected cells (Fig. 4A). In contrast, the ratio of basal cAMP levels in cells expressing *Am*DOP2 versus uninfected cells, was close to 1 (1.3±0.1 S.E.M.). Although variation in the extent of elevation of cAMP levels in Sf21 cells expressing *Am*DOP1 was observed, in a majority (85%) of assays, cells expressing *Am*DOP1 receptors exhibited basal levels of cAMP that were 1.5-fold or higher than those determined in either cells expressing the *Am*DOP2 receptor, or uninfected cells (Fig. 4A). A similar trend was apparent in Sf9 cells (Fig. 4B). In this cell line, however, enhancement of basal levels of cAMP in cells expressing the *Am*DOP1 receptor was less pronounced. On average, basal cAMP levels were 1.7±0.3 (S.E.M.)-fold higher in Sf9 cells expressing *Am*DOP1 receptors than in uninfected cells, while basal cAMP levels in cells expressing *Am*DOP2 receptors were slightly lower than in uninfected cells (0.81±0.08).

In Sf21 cells expressing *Am*DOP1 receptors, basal levels of cAMP were reduced by flupentixol to levels not

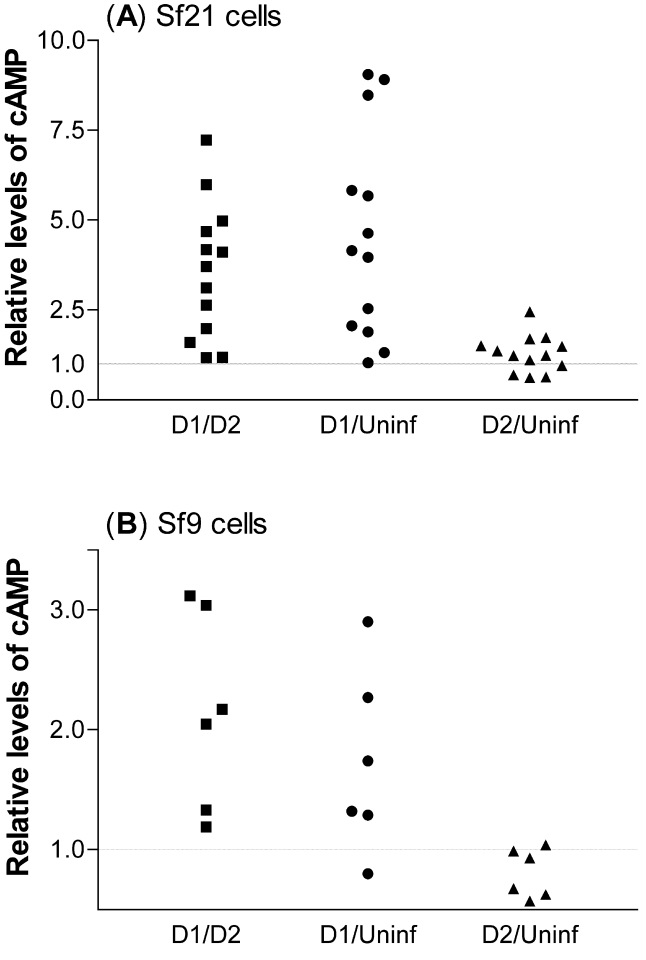

Fig. 4. Relative levels of intracellular cAMP in cells expressing *Am*DOP1 receptors, *Am*DOP2 receptors, or neither receptor (uninfected cells). Basal levels of cAMP (measured in 100 μM IBMX) determined for cells expressing *Am*DOP1 receptors (D1), *Am*DOP2 receptors (D2) and uninfected cells (Uninf) are presented as ratios. (A) Relative basal levels of cAMP were determined for 13 independent assays conducted with Sf21 cells. (B) Relative levels of cAMP for six independent assays using Sf9 cells. Although the magnitude of the elevation in cAMP varied from assay to assay, the ratio of basal cAMP levels in cells expressing *Am*DOP1 receptors versus basal levels in uninfected cells (D1/Uninf) is significantly different from the ratio of basal cAMP levels in cells expressing *Am*DOP2 receptors versus uninfected cells (D2/Uninf) as determined by a paired, two-tailed, *t*-test. (For Sf21 cells: $P=0.0006$, $t=4.6$, df = 12. For Sf9 cells: $P=0.021$, $t=3.0$, df = 5.)

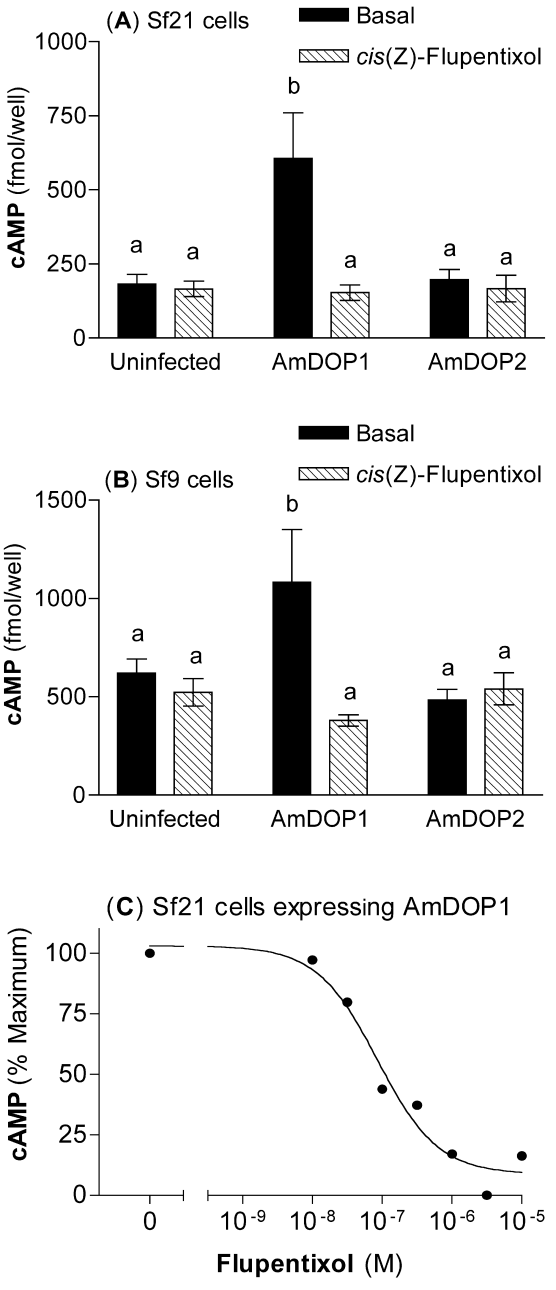

Fig. 5. Effect of flupentixol on basal cAMP levels in cells expressing the *Am*DOP2 receptor, the *Am*DOP1 receptor, or neither receptor (uninfected cells). (A) Sf21 cells or (B) Sf9 cells were treated with either, IBMX alone (Basal), or with IBMX plus 10^{-5} M *cis*(*Z*)-flupentixol. Values shown are means ± S.E.M. for six independent experiments. Overall statistical significance was determined by one-way ANOVA followed by Tukey–Kramer tests. Letters over each bar on the graph indicate whether or not differences between groups are significant. Groups with the same letter are not significantly different. (For Sf21 cells: $F=6.22$; $P=0.0005$; for Sf9 cells: $F=3.88$, $P=0.0081$.) (C) Treatment of Sf21 cells expressing *Am*DOP1 receptors with IBMX plus a range of flupentixol concentrations. The point labeled '0' indicates the cAMP level measured with IBMX alone (the basal level). The curve shown is representative of three independent experiments done in duplicate.

significantly different from those observed in uninfected cells, and in cells expressing the *Am*DOP2 receptor (Fig. 5A). Treatment of Sf9 cells expressing *Am*DOP1 receptors with flupentixol also reduced basal cAMP to a level similar to those observed in cells expressing *Am*DOP2, and in uninfected cells (Fig. 5B). Sf21 cells expressing *Am*DOP1 receptors were treated with a range of flupentixol concentrations revealing that flupentixol reduces basal cAMP levels in a dose-dependent manner (Fig. 5C). Compounds that reduce increases in unstimulated 'baseline' activity (in this case, basal cAMP levels), have been referred to elsewhere as 'negative antagonists' or 'negative agonists'.

However, the term that is now most commonly used to describe such compounds is ‘inverse agonists’ (reviewed in Ref. [5]).

In all three assays in which the effects of dopamine on cells expressing *Am*DOP1 and *Am*DOP2 were examined in parallel, dopamine was found to be more potent at stimulating cAMP production via *Am*DOP1 receptors than via *Am*DOP2 receptors (Fig. 6A, see also Table 1). The dopamine receptor agonist 6,7-ADTN was also more potent at *Am*DOP1 receptors than *Am*DOP2 (Fig. 6B and Table 1). Direct comparison of the ability of flupentixol, butaclamol, spiperone and SCH 23390 to block dopamine-mediated stimulation of cAMP in cells expressing *Am*DOP1 (Fig. 7A) or *Am*DOP2 (Fig. 7B) revealed that at both receptors, flupentixol was the most potent of the antagonists tested, followed by butaclamol, spiperone and then SCH 23390 (Table 1).

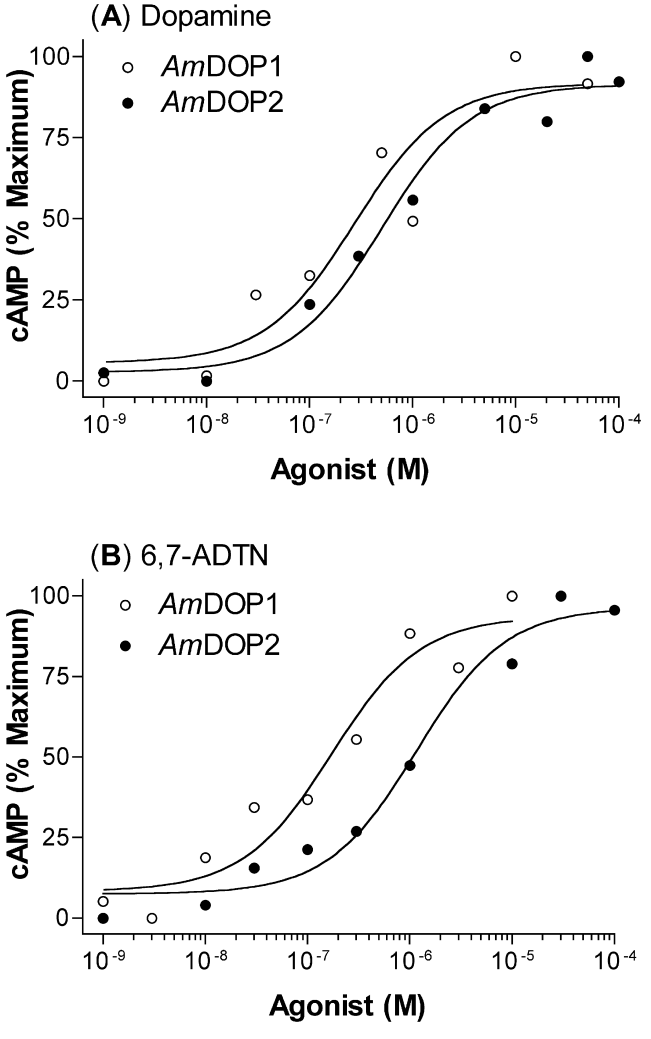

Fig. 6. Agonist response curves for Sf21 cells expressing either *Am*DOP1 or *Am*DOP2 receptors. For these experiments, cells expressing *Am*DOP2 receptors were examined in parallel with cells expressing *Am*DOP1 receptors. To allow comparison, the data have been normalized with the minimum cAMP level set to zero and the maximum to 100% for each curve. Representative curves are shown for three independent experiments done in duplicate. (A) Curves illustrating the change in cAMP levels due to treatment with a range of dopamine concentrations. (B) Representative curves for the response of cells expressing each receptor to 6,7-ADTN at the concentrations indicated. The mean EC_{50} values from independent experiments are reported in Table 1.

Table 1
Comparison of mean EC_{50} and IC_{50} values of different compounds for cells expressing *Am*DOP1 or *Am*DOP2 receptors

	Ligand	*Am*DOP1	*Am*DOP2
EC_{50} in μM (pEC_{50}±SEM, *n*)	Dopamine	0.36 (6.44±0.08, 3)	2.2 (5.66±0.19, 6)
	6,7-ADTN	0.65 (6.19±0.23, 3)	5.1 (5.29±0.44, 3)
IC_{50} in μM[a] (pIC_{50}±SEM, *n*)	*cis*(*Z*)-Flupentixol	0.20 (6.71±0.15, 3)	0.00380 (8.42±0.02, 3)
	(+)-Butaclamol	0.54 (6.27±0.05, 3)	0.081 (7.09±0.32, 3)
	Spiperone	2.2 (5.66±0.03, 3)	8.5 (5.07±0.07, 3)
	R(+)-SCH23390	8.1 (5.09±0.09, 3)	17 (4.78±0.48, 3)

[a] IC_{50} values were determined in the presence of 1 μM dopamine for assays with *Am*DOP1 and 10 μM dopamine for assays with *Am*DOP2.

4. Discussion

Direct comparison of *Am*DOP1 and *Am*DOP2 receptors revealed that, in a majority of assays, Sf21 or Sf9 cells expressing *Am*DOP1 contained higher basal levels of cAMP than either cells expressing *Am*DOP2, or uninfected cells. These results suggest that the *Am*DOP1 receptor exhibits agonist-independent activation. Interestingly, a recent review of G protein-coupled receptors (GPCR) reported that more than 40% of all characterized GPCRs are constitutively active [41]. Although Northern analysis does not provide for the direct determination of receptor levels, analysis of cells expressing *Amdop1* or *Amdop2* revealed that *Amdop2* was expressed at slightly higher levels than *Amdop1* (Fig. 3). While variations in *Am*DOP1 receptor density may contribute to the variations in basal levels of cAMP (Fig. 4), it seems unlikely that constitutive activity of *Am*DOP1 is an artifact arising from the expression of high levels of *Am*DOP1 receptors. There are several lines of evidence that support this argument. Firstly, expression of similar high levels (above physiological levels) of G protein-coupled receptors have demonstrated that, under the same conditions, some receptors have high levels of constitutive activity while others show little or no agonist independent activity [7,42,47] (see Ref. [41] for a review). Secondly, GPCRs that have been shown to be constitutively active in heterologous expression systems have been found to be constitutively active also, when analyzed at physiological levels in their native tissues [16,31]. Thirdly, GPCRs that are not constitutively activated in recombinant expression systems, remain not

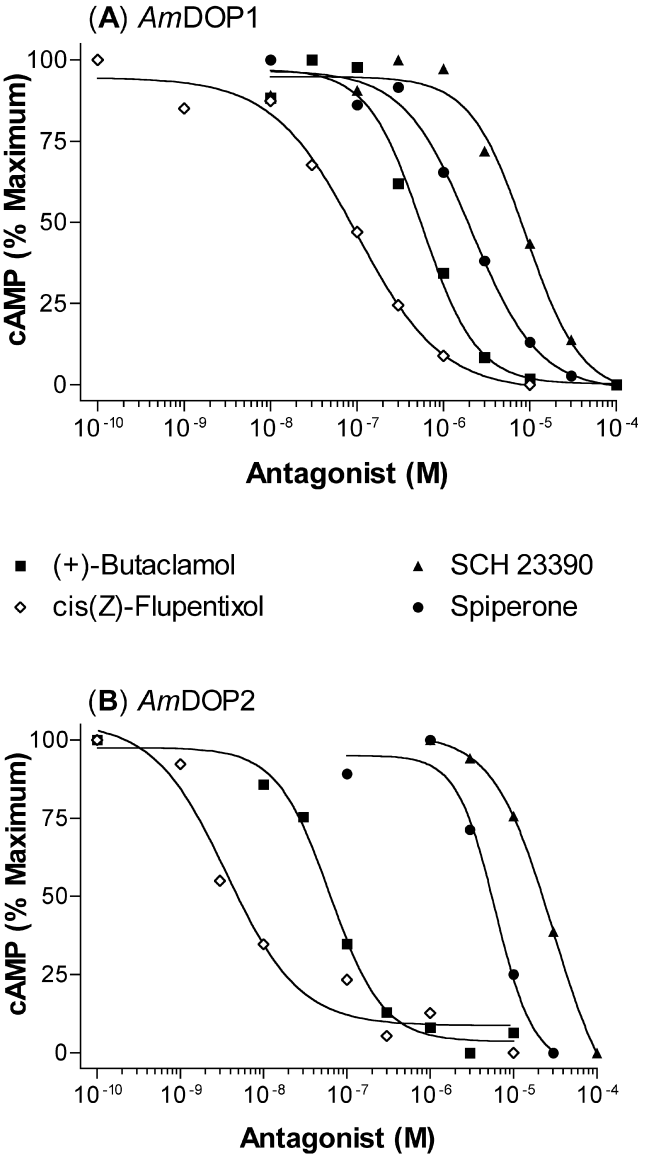

Fig. 7. Effects of dopamine receptor antagonists acting via *Am*DOP1 receptors (A) or *Am*DOP2 receptors (B). (A) Cells expressing *Am*DOP1 receptors were exposed to dopamine (10^{-6} M) and the selected antagonist at the range of concentrations indicated. (B) Cells expressing *Am*DOP2 receptors were treated with 10^{-5} M dopamine and the antagonist indicated. For comparison, the data have been normalized so that for each curve, the maximum cAMP level determined was set at 100% and the minimum level is zero. Representative curves are shown from three experiments done in duplicate. The mean IC_{50} values derived from these experiments are shown in Table 1.

constitutively activated in their native systems, even when expressed many times over their physiological levels [50]. Taken together, these data argue that constitutive activity is an intrinsic feature of some receptors, and is not an artifact due to over expression in recombinant systems. In this context, it is significant, that despite the fact that *Amdop1* and *Amdop2* were driven off the same, very strong promoter, *Am*DOP1 receptors exhibited constitutive activity, whereas *Am*DOP2 receptors did not.

Constitutive activation of adenylyl cyclase by a dopamine receptor is not unique to the *Am*DOP1 receptor. Like *Am*DOP1, the vertebrate D5/D1B receptor is also constitutively activated [47], a property that serves to differentiate between mammalian D1/D1A and D5/D1B receptors. Agonist-independent activities have also been associated with D1-like dopamine receptors in nonmammalian vertebrates, such as eel [7] and frog [44], and recent evidence suggests that a *C. elegans* dopamine receptor may also exhibit this property (Sanyal and Van Tol, personal communication), but the present study is the first to identify such a property in an insect dopamine receptor.

While the physiological relevance of agonist-independent receptor activity remains unknown, higher levels of intracellular cAMP resulting from expression of a constitutively activated receptor may have a significant impact on the physiological properties of a cell. For example, cAMP activates the cAMP-dependent protein kinase, protein kinase A, which in turn phosphorylates target proteins that can include ion channels, as well as proteins involved in regulation of gene expression. The increase in basal levels of cAMP in cells expressing constitutively activated receptors such as *Am*DOP1 or D5/D1B, lowers the signal-to-noise ratio, an observation that has led to the suggestion that constitutively active receptors may act as on/off switches [24]. The possibility that endogenous ligands may exist that mimic the effects of inverse agonists, such as flupentixol (see Fig. 5), in reducing the agonist-independent activity of constitutively activated receptors is also of great interest, and may be highly significant in terms of the functional properties of such receptors. While endogenous inverse agonists have yet to be identified for biogenic amine receptors, they have been described for other G protein-coupled receptors. For example, the endogenous agouti and agouti-related proteins act as inverse agonists at melanocortin receptors [32,43].

The structural basis of the constitutive activity of dopamine receptors has been investigated in a number of studies [8,10,19,20]. The construction of chimeric receptor proteins, in which the carboxyl-terminal tail sequence of the D5/D1B receptor was exchanged with that of the D1/D1A receptor, has identified a region of the carboxyl-terminus located ~70 residues downstream from the seventh transmembrane region as being important for constitutive activity of the human D5/D1B receptor [10]. Interestingly, the carboxyl tail of the *Am*DOP1 receptor is shorter than the human D5/D1B receptor (71 amino acids versus 113) and does not contain the corresponding region. A residue in the third cytoplasmic loop has also been implicated in partially modulating the constitutive activity of D5/D1B [8]. The residue is isoleucine in the D5/D1B receptor, and phenylalanine in the D1/D1A, and in the *Am*DOP1 receptor, the corresponding residue is a histidine. Such comparisons suggest that the constitutive activity of *Am*DOP1 may be conferred via a different structural mechanism than for vertebrate dopamine receptors.

In addition to the agonist-independent activity of *Am*DOP1, a direct comparison of *Am*DOP1 and *Am*DOP2 receptors reveals differences between these two honey bee dopamine receptors that parallel differences between vertebrate D1/D1A and D5/D1B receptors. For example, the *Am*DOP1 receptor reaches maximal activation with about 10-fold lower concentrations of dopamine and 6,7-ADTN than *Am*DOP2. In vertebrates also, the constitutively activated D1 receptor, D5/D1B, has a 10-fold higher affinity for dopamine than the D1/D1A receptor. The antagonists flupentixol and butaclamol, on the other hand, have a higher affinity for D1/D1A than D5/D1B receptors. In a striking parallel, these two antagonists appeared also to be more potent at blocking responses mediated via *Am*DOP2 receptors than the constitutively activated receptor, *Am*DOP1. These results suggest that although the *Am*DOP1 and *Am*DOP2 receptors have a relatively low level of homology compared to the D1/D1A and D5/D1B receptors, there may be functional parallels between these honey bee and mammalian receptors. Evidence suggests that *Am*DOP1 receptors are expressed widely throughout the brain of the honey bee [3] whereas *Am*DOP2 receptors are restricted mainly to the mushroom bodies of the protocerebrum [17]. In the mammalian brain, however, the D1/D1A receptor is expressed more widely than the constitutively activated receptor, D5/D1B [30,48].

This study is the first to provide evidence for a constitutively activated invertebrate dopamine receptor. Although phylogenetic analysis shows that, unlike the vertebrate D1/D1A and D5/D1B receptors, *Am*DOP1 and *Am*DOP2 are not closely related paralogs [17,22], it is significant that this property is conserved between insects and mammals. The direct comparison between *Am*DOP1 and *Am*DOP2 has shown that although both receptors upregulate intracellular cAMP levels in the presence of dopamine, the two receptors have functional properties that enable them to be clearly differentiated.

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Der Tyramin-Rezeptor der Honigbiene – AmTYR1

- **BLENAU, W.**, BALFANZ, S. AND BAUMANN, A. (2000) Am*tyr*1: characterization of a gene from honeybee (*Apis mellifera*) brain encoding a functional tyramine receptor. *J. Neurochem.* **74**(3), 900-908.
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Amtyr1: Characterization of a Gene from Honeybee (*Apis mellifera*) Brain Encoding a Functional Tyramine Receptor

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Abstract: Biogenic amine receptors are involved in the regulation and modulation of various physiological and behavioral processes in both vertebrates and invertebrates. We have cloned a member of this gene family from the CNS of the honeybee, *Apis mellifera.* The deduced amino acid sequence is homologous to tyramine receptors cloned from *Locusta migratoria* and *Drosophila melanogaster* as well as to an octopamine receptor cloned from *Heliothis virescens.* Functional properties of the honeybee receptor were studied in stably transfected human embryonic kidney 293 cells. Tyramine reduced forskolin-induced cyclic AMP production in a dose-dependent manner with an EC_{50} of ~130 n*M.* A similar effect of tyramine was observed in membrane homogenates of honeybee brains. Octopamine also reduced cyclic AMP production in the transfected cell line but was both less potent (EC_{50} of ~3 μM) and less efficacious than tyramine. Receptor-encoding mRNA has a widespread distribution in the brain and subesophageal ganglion of the honeybee, suggesting that this tyramine receptor is involved in sensory signal processing as well as in higher-order brain functions. **Key Words:** Cyclic AMP—G protein-coupled receptor—Invertebrate—Octopamine—Phenolamine—Stable transformation.
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Tyramine and octopamine are monophenolic amines that belong to a group of compounds known as biogenic amines. High concentrations of both amines are found in the CNS of invertebrates, whereas only trace amounts have been detected in vertebrate brains (Axelrod and Saavedra, 1977; David and Coulon, 1985; Osborne, 1996). Based on this phylogenetic difference it is assumed that the sympathetic system of vertebrates is functionally substituted by the tyraminergic/octopaminergic system in invertebrates (Evans, 1985, 1993).

Biogenic amines bind to membrane proteins that are usually members of the superfamily of GTP-binding protein (G protein)-coupled receptors and share the structural motif of seven transmembrane (TM) domains. Activation of these receptors leads to changes in the concentration of intracellular second messengers, i.e., cyclic nucleotides [cyclic AMP (cAMP) and cyclic GMP], inositol trisphosphate, and Ca^{2+}. Biochemical and pharmacological studies in different tissues revealed that both tyramine and octopamine induce changes in the intracellular concentration of cAMP or Ca^{2+} ($[cAMP]_i$ and $[Ca^{2+}]_i$, respectively) (Evans, 1980, 1984, 1985; Uzzan and Dudai, 1982).

Many behavioral responses and physiological reactions were attributed to the action of octopamine (Evans, 1980, 1985; David and Coulon, 1985; Erber et al., 1993; Orchard et al., 1993), and current hypotheses suggest that an octopamine-induced increase of $[cAMP]_i$ is part of a molecular mechanism underlying learning and memory in insects (Hildebrandt and Müller, 1995; Meller and Davis, 1996; Hammer, 1997; Han et al., 1998). In contrast to octopamine, the physiological role of tyramine is less well understood. However, both amines often mediate similar behavioral effects. In the honeybee, the proboscis extension reflex is an appetitive component of the animal's feeding behavior. The reflex can be elicited experimentally by touching either antenna with a droplet of sugar water. When animals are treated with reserpine, which inhibits vesicular uptake of biogenic amines, the nervous system becomes depleted of monoamines (Sloley and Owen, 1982; Brookhart et al., 1987). Under these conditions, either the proboscis extension reflex is completely suppressed, or the responsiveness of the animal to the stimulus is significantly impaired (Braun and

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Abbreviations used: $[Ca^{2+}]_i$, intracellular concentration of Ca^{2+}; cAMP, cyclic AMP; $[cAMP]_i$, intracellular concentration of cyclic AMP; G protein, GTP-binding protein; HEK, human embryonic kidney; PKA, cyclic AMP-dependent protein kinase; PKC, protein kinase C; PKG, cyclic GMP-dependent protein kinase; SSC, saline–sodium citrate; TM, transmembrane.

Bicker, 1992). In ~50% of the reserpinized bees, the reflex is restored when either octopamine or tyramine is injected into the hemolymph (Braun and Bicker, 1992). Injection of other biogenic amines, i.e., dopamine or serotonin, does not restore the proboscis extension reflex. Although the effect of octopamine is diminished within 1 h, the effect of tyramine persists for ≥2 h. This result led Braun and Bicker (1992) to propose that octopamine might be subject to a faster enzymatic breakdown. However, tyramine also accelerated the rate of habituation of the reflex, when it was applied to nonreserpinized animals (Braun and Bicker, 1992). These data suggest that in the honeybee, tyramine and/or octopamine are involved in mediating food arousal, the state dependence of habituation, and the response decrement during habituation. It was assumed that the effects of tyramine were mediated by the activation of octopamine receptors either after biochemical conversion of tyramine to octopamine or by direct binding of tyramine to octopamine receptors, rather than by activation of specific tyramine receptors (Braun and Bicker, 1992), suggesting that tyramine receptors might not necessarily exist. Nevertheless, there is good pharmacological evidence that different binding sites for [^{3}H]tyramine and [^{3}H]octopamine do exist in the locust brain (Hiripi et al., 1994). These results have been further substantiated by molecular cloning of cDNA encoding a tyramine receptor from *Locusta migratoria* (Vanden Broeck et al., 1995). A related receptor sequence had already been isolated from *Drosophila* (Arakawa et al., 1990; Saudou et al., 1990). Expression of the genes in different cell lines led to a tyramine-dependent inhibition of cAMP synthesis (Saudou et al., 1990; Robb et al., 1994; Vanden Broeck et al., 1995). The *Drosophila* receptor has also been shown to display "agonist-specific coupling" to multiple second messenger systems in Chinese hamster ovary cells (Robb et al., 1994). Although tyramine is about two orders of magnitude more potent than octopamine in inhibiting forskolin-stimulated cAMP accumulation, octopamine is slightly more potent in causing a transient elevation of $[Ca^{2+}]_i$ (Robb et al., 1994).

To gain further insight into the function of tyramine, we decided to clone and characterize tyramine receptors from the honeybee (*Apis mellifera*), an organism in which learning and memory have been studied quite extensively (for recent reviews, see Hammer and Menzel, 1995; Meller and Davis, 1996; Menzel and Müller, 1996). Several cDNA clones were isolated that share high sequence homology with tyramine receptors from *Locusta* (Vanden Broeck et al., 1995) and *Drosophila* (Saudou et al., 1990). In stably transfected human embryonic kidney (HEK) 293 cells, activation of the receptor (AmTYR1) inhibits forskolin-stimulated cAMP synthesis with an EC_{50} of ~130 n*M*. Octopamine is both less potent (EC_{50} of ~3 μM) and less efficacious than tyramine. In membrane fractions from honeybee brains, tyramine also inhibited cAMP synthesis, suggesting that cloned and native tyramine receptors activate the same signaling pathway. In situ hybridization showed that the mRNA encoding AmTYR1 is abundantly expressed in the brain of adult worker honeybees. The characterization of the AmTYR1 receptor will facilitate further analyses examining the functional contribution of the tyraminergic system in the honeybee at both the behavioral and physiological levels.

MATERIALS AND METHODS

Materials

[α-^{32}P]dCTP (specific activity, >3,000 Ci/mmol) and the cAMP assay kit (TRK 432) were from Amersham (Germany). [^{3}H]cAMP (specific activity, 33 Ci/mmol) and [α-^{32}P]ATP (specific activity, 20–40 Ci/mmol) were from DuPont (New England Nuclear, Germany). Neurochemicals were purchased from RBI and Sigma. Digoxigenin-labeled UTP was from Boehringer (Germany). Nylon membranes were from Qiagen (Germany).

Isolation of cDNA clones and sequencing

We screened 1×10^6 λ phages of a cDNA library from *A. mellifera* brains (Blenau et al., 1998) using the complete cDNA encoding a tyramine receptor from *Drosophila* as a probe. Hybridization was performed as described (Blenau et al., 1998). Positive recombinants were identified by Southern blotting and sequencing. A restriction fragment of one recombinant was used to rescreen the library at high stringency. In total, 26 recombinants were proven as positive clones by Southern blotting. DNA fragments were labeled with [α-^{32}P]dCTP using a DECAprime kit (Ambion, U.S.A.). Plasmid DNA of positive clones was isolated following the in vivo excision protocol (Stratagene, U.S.A.). Subcloning of restriction fragments was done by standard cloning techniques (Sambrook et al., 1989). Sequencing of cDNA clones was performed with a Thermo Sequenase fluorescent-labeled primer cycle sequencing kit (RPN 2538; Amersham). Probes were analyzed on a LICOR electrophoresis system (MWG Biotech, Germany). The nucleotide sequence of Am*tyr*1 has been submitted to the EBI database under accession no. AJ245824.

In situ hybridization

Hybridization to cryosections of adult worker honeybee brains was performed with digoxigenin-labeled riboprobes. Antisense and sense probes were transcribed from a subclone encoding the third cytoplasmic loop of the receptor (bp 884–1,026) in pBluescript SK(−) vector using T7 and T3 RNA polymerase, respectively. Hybridization was in 50% formamide, 5 × saline–sodium citrate (SSC; 20 × SSC is 3 *M* NaCl and 0.3 *M* sodium citrate, pH 7.4), 100 μg/ml autoclaved herring testes DNA, 50 μg/ml heparin, and 0.1% Tween 20 at 42°C for 12 h. Washing was done three times in 50% formamide and 1 × SSC at 37°C for 1 h each. For detection of hybrids, we followed the digoxigenin application manual of Boehringer (Germany).

Construction of pcAm*tyr*1 expression vector

A truncated version of Am*tyr*1 cDNA containing a Kozak consensus sequence (Kozak, 1984) immediately 5′ to the ATG codon of the open reading frame was constructed by PCR. The oligonucleotide 5′-GGCAAGCTTCCACCATGAACTCGAGCGGG was used as the 5′ primer, and the oligonucleotide 5′-GGCGGCAAATCTTGTT (bp 1,362–1,378) was used as the 3′ primer. The PCR product was digested with *Hin*dIII and *Bam*HI. Am*tyr*1 cDNA in pBluescript SK(−) vector was digested with *Bam*HI and *Eco*RI. Restriction fragments were

gel-purified and ligated into *Hin*dIII- and *Eco*RI-cut pcDNAI-amp vector (Invitrogen, U.S.A). The PCR-generated part of this clone, pcAm*tyr*1, was verified by sequencing. The plasmid was cut with *Hin*dIII and *Xba*I, and the cDNA was subcloned into pcDNAIneo vector (Invitrogen, U.S.A).

Functional expression of AmTYR1 receptors

Approximately 10 μg of the Am*tyr*1 recombinant in pcDNAIneo vector was introduced into exponentially growing ($\sim 2 \times 10^5$ cells per 50-mm-diameter dish) HEK 293 cells by a modified calcium phosphate method (Chen and Okayama, 1987). Transfected clones were selected in the presence of the antibiotic G418 at 1.0 mg/ml. Isolated foci were propagated and analyzed for expression of AmTYR1 receptors, considering that activation of AmTYR1 leads to an inhibition of adenylyl cyclase activity in these cells. Assays to determine cAMP concentrations were performed as described earlier (Gotzes and Baumann, 1996). Adenylyl cyclase was activated with 20 μM forskolin. The reduction of cAMP levels after treatment with different biogenic amines is expressed as a percentage of the value determined with forskolin (defined as 100%). Mean values of cAMP concentrations per dish were determined from at least three independent experiments conducted in duplicate. Data were processed by using the PRISM program (GraphPad).

The experimental details to determine $[Ca^{2+}]_i$ in HEK 293 cells using fluorescence imaging of the Ca^{2+} indicator dye fura-2 have been described previously (Baumann et al., 1994). Concentrations of biogenic amines used for activation of the AmTYR1 receptor are given in the text.

Determination of adenylyl cyclase activity in membrane fractions of honeybee brains

Brains of worker bees were dissected in ice-cold Ringer's solution (270 m*M* NaCl, 3.2 m*M* KC1, 1.2 m*M* $CaCl_2$, 10 m*M* $MgCl_2$, and 10 m*M* morpholinopropanesulfonic acid, pH 7.3). The tissue was homogenized at 4°C in buffer 1 (10 m*M* Tris-HC1, 1 m*M* EGTA, and 4 m*M* $MgCl_2$, pH 7.4) and centrifuged for 10 min at 40,000 *g*. The pellets were rehomogenized and centrifuged (see above). Finally, the pellets were homogenized in buffer 2 (10 m*M* Tris-HC1 and 1 m*M* EGTA, pH 7.4) and stored frozen at −80°C until further use.

The effect of biogenic amines on adenylyl cyclase activity in these membrane fractions was measured as described by Schultz and Jakobs (1984). Assays were performed for 10 min at 25°C in 50 m*M* Tris-HC1 (pH 7.4), 1 m*M* $MgCl_2$, 30 μM GTP, 1 m*M* 3-isobutyl-1-methylxanthine, 100 μM cAMP, 100 μM ATP, 5 m*M* creatine phosphate, 1.52 kU of creatine kinase, 1 mg/ml bovine serum albumin, 200,000–300,000 dpm of [α-^{32}P]ATP, and 30 μl of membrane homogenate (9–14 μg of protein) in a total volume of 100 μl. Adenylyl cyclase activity was tested in the presence of 10 μM forskolin (defined as 100%) and different concentrations of either tyramine or octopamine. Reactions were terminated by addition of 400 μl of 125 m*M* $ZnCl_2$ and 500 μl of 125 m*M* Na_2CO_3. The synthesized [^{32}P]cAMP was isolated by chromatography on $A1_2O_3$ and monitored by liquid scintillation spectroscopy.

RESULTS

Molecular and structural properties of a tyramine receptor cloned from honeybee brain

Several cDNA clones encoding putative tyramine receptors were isolated form a cDNA library of honeybee brain (see Materials and Methods). One recombinant (Am*tyr*1) that contained an insert of ~4.0 kb was analyzed in detail.

The Am*tyr*1 cDNA consists of 4,195 bp. The translation initiation codon (nucleotides 111–113 of cloned cDNA) was assigned to the first ATG codon of the longest open reading frame. Nonsense codons were found in all three reading frames preceding this ATG codon. The open reading frame of Am*tyr*1 is terminated by a translational stop codon TGA at positions 1,308–1,310. The deduced amino acid sequence (AmTYR1) of the open reading frame consists of 399 residues with a calculated relative molecular mass of 44,650. The 3′ noncoding region of the cDNA consists of 2,885 bp and is terminated by a poly(dA) tract of 16 residues.

The deduced amino acid sequence of Am*tyr*1 (Fig. 1A) shares many of the characteristic features of G protein-coupled receptors. The hydropathy profile predicts seven putative membrane spanning segments (TM1–TM7; see Fig. 1B). One consensus site for N-linked glycosylation (N_2SS) is located in the N terminus of AmTYR1 (see Fig. 1A). One consensus site for phosphorylation by cAMP/cyclic GMP-dependent protein kinases (PKA/PKG, respectively) is located at position (T_{296}), and seven phosphorylation sites for protein kinase C (PKC) were identified within the intracellular loops at positions T_{63}, T_{149}, T_{223}, S_{241}, T_{265}, and $S_{291/292}$ (see Fig. 1A). Whether phosphorylation of any of these residues participates in receptor desensitization during longer exposure to ligands remains to be experimentally examined.

A high degree of homology exists between AmTYR1 and cloned tyramine receptors from *Drosophila melanogaster* [DmTYR (Arakawa et al., 1990; Saudou et al., 1990)] and *L. migratoria* [LocTYR (Vanden Broeck et al., 1995)] as well as between AmTYR1 and an octopamine receptor cloned from the moth *Heliothis virescens* [K50Hel = HelOCT (von Nickisch-Rosenegk et al., 1996)]. The overall amino acid similarity (identical and conservative amino acid substitutions) among AmTYR1, DmTYR, and LocTYR is 80.2 and 83.2%, respectively. The amino acid similarity between AmTYR1 and HelOCT is 78.2%. An alignment of these receptor sequences is shown in Fig. 2A. Sequence conservation to other biogenic amine receptors from invertebrates is less pronounced. A phylogenetic analysis of AmTYR1 with different biogenic amine receptor sequences showed that AmTYR1, DmTYR, LocTYR, and HelOCT form a distinct subgroup within the family of invertebrate biogenic amine receptors (Fig. 2B).

Amino acid residues that are highly conserved between members of the biogenic amine receptor family are also found in AmTYR1. An aspartic acid residue (D_{116}) in TM3 (see Fig. 1A) most likely binds to the protonated amino group of the ligand. A sequence motif of regularly spaced serine residues in TM5 (SSXXS; S = serine, X = any amino acid) that is characteristic for catecholaminergic receptors is also present in AmTYR1 (S_{200}SLGS; see Fig. 1A). These serine residues have been shown to interact with the hydroxyl groups of the

A

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                                                                TM 1
MNSSG ESGGT MTEDY DMTGC GPPEE ETGSN LPVWE AAAAS LTLGF LVLAT   50
                  ▽                 TM 2
VLGNA LVILS VFTYR PLRIV QNFFI VSLAV ADLAV AILVM PFNVA YLLLG  100
                        TM 3                          ▽
KWIFG IHLCK LWLTC DVLCC TASIL NLCAI ALDRY WAITD PINYA QKRTL  150
          TM 4
KRVLA TIAGV WILSG AISSP PLAGW NDWPE ELEPG TPCQL TRRQG YVIYS  200
          TM 5                  ▽                 ▽
SLGSF FIPLL LMSLV YLEIY LATRR RLRER ARQSR INAVQ STRHR EADDA  250
                ▽                                 ▽▽    ▽
EESVS SETNH NERST PRSHA KPSLI DDEPT EVTIG GGGTT SSRRT TGSRA  300
                                        TM 6
AATTT TVYQF IEERQ RISLS KERRA ARTLG VIMGV FVVCW LPFFL MYVIV  350
                        TM 7
PFCPD CCPSD RMVYF ITWLG YVNSA LNPLI YTIFN LDYRR AFRRL LRIR   399
```

B

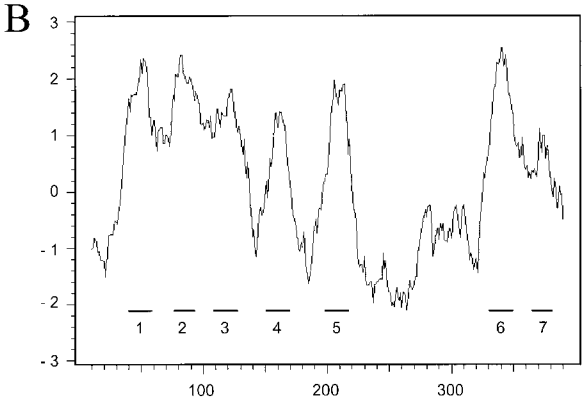


FIG. 1. A: Deduced amino acid sequence of AmTYR1. Amino acids (in one letter code) are numbered beginning with the initiating methionine. The position of the last residue in each line is indicated at the right. The putative transmembrane regions TM1–TM7 are overlined. A potential *N*-glycosylation site is indicated (●). Potential phosphorylation sites for PKC and PKA/PKG are indicated (▽). **B:** Hydropathy profile of AmTYR1. The profile was calculated according to the algorithm of Kyte and Doolittle (1982) using a window size of 19 amino acids. Amino acid numbers are given on the abscissa; hydrophobicity values are given on the ordinate. The putative TM regions are indicated by horizontal bars and numbered 1–7.

benzoyl ring of catecholaminergic ligands (Strader et al., 1989, 1995). The C terminus of AmTYR1 is very short and consists of only 16 amino acids. It lacks cysteine residues that could be the target for palmitoylation, a posttranslational modification that frequently occurs in other G protein-coupled receptors.

Functional expression of Am*tyr*1 cDNA in HEK 293 cells

A cell line that stably expressed AmTYR1 receptors was generated (see Materials and Methods). This cell line was examined for the functional coupling of AmTYR1 to intracellular effector systems. Stimulation with tyramine (10^{-6} *M*) elicited an increase of neither $[Ca^{2+}]_i$ nor $[cAMP]_i$ (data not shown).

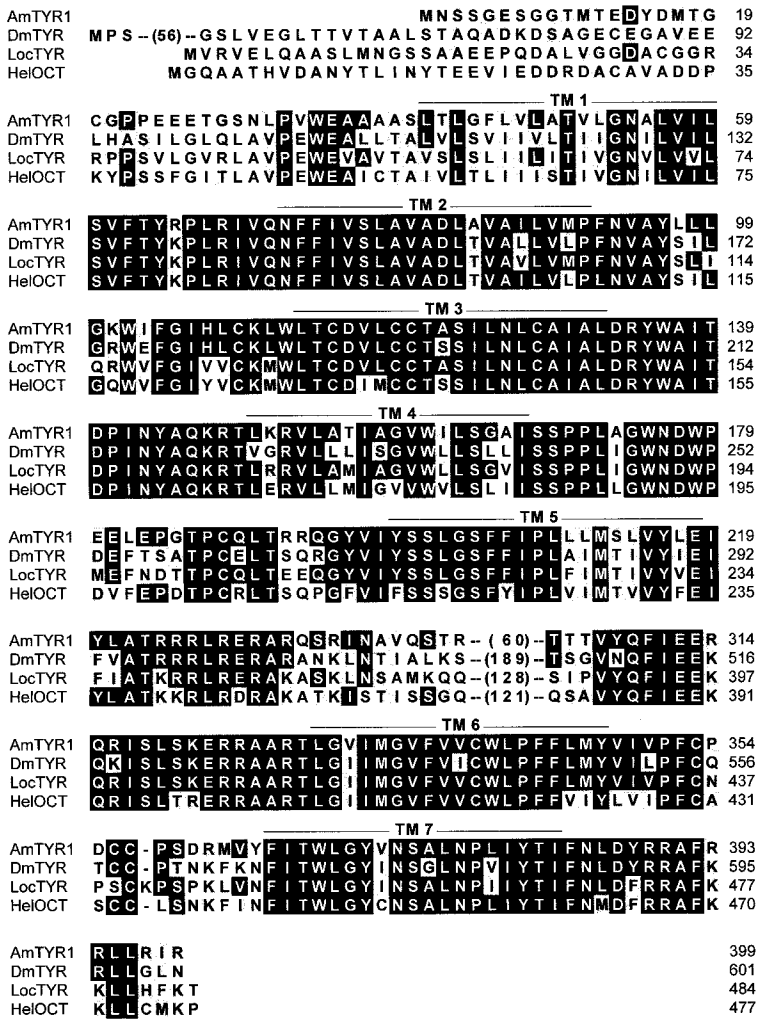
Forskolin, a direct activator of adenylyl cyclase, stimulates synthesis of cAMP in HEK 293 cells (Gotzes et al., 1994). In the cell line that stably expressed AmTYR1 receptors, tyramine attenuated the forskolin-induced production of cAMP in a dose-dependent manner. Half-maximal reduction of cAMP levels (EC_{50}) was observed with ~130 n*M* tyramine ($\log EC_{50} = -6.89 \pm 0.23$, mean ± SE), but cAMP synthesis was maximally reduced by ~30% (27.5 ± 2.4%, mean ± SE) with tyramine concentrations of ≥50 μM (Fig. 3). Octopamine also reduces cAMP production in AmTYR1-expressing cells. The maximal reduction of cAMP synthesis, however, was ~10% (7.7 ± 4.0%, mean ± SE), and the EC_{50} value (~3 μM; $\log EC_{50} = -5.56 \pm 0.65$, mean ± SE) is ~20-fold greater compared with tyramine (Fig. 3). Similar properties have been described for a tyramine receptor from *Drosophila* (Saudou et al., 1990).

We also tested the effect of dopamine and serotonin on AmTYR1-expressing cells. Dopamine (10^{-6} *M*) did not elicit any cellular response (data not shown). In contrast, serotonin (10^{-6} *M*) led to a substantial decrease of forskolin-induced cAMP synthesis that reached a similar value as that observed with tyramine. However, the serotonin-dependent inhibition of adenylyl cyclase activity is caused by the activation of endogenous serotonin receptors rather than the AmTYR1 receptor because we observed the same effect on nontransfected HEK 293 cells. For the other biogenic amines tested, i.e., dopamine (10^{-6} *M*), octopamine (10^{-6} *M*), and tyramine (10^{-6} *M*), nontransfected HEK 293 cells responded with a change of neither $[cAMP]_i$ nor $[Ca^{2+}]_i$ (data not shown).

Effects of tyramine and octopamine on cAMP production in membrane homogenates of honeybee brains

We studied the effects of tyramine and octopamine on adenylyl cyclase activity in membrane homogenates of honeybee brain. Adenylyl cyclase was stimulated with 10 μM forskolin (defined as 100%). When membrane fractions were coincubated with 0.1–1 μM tyramine, cAMP synthesis was attenuated by ~25% (Fig. 4). This value is very similar to the inhibitory effect determined on the AmTYR1-expressing cell line (Fig. 3). At a concentration of 10 μM tyramine, the inhibitory effect was

A



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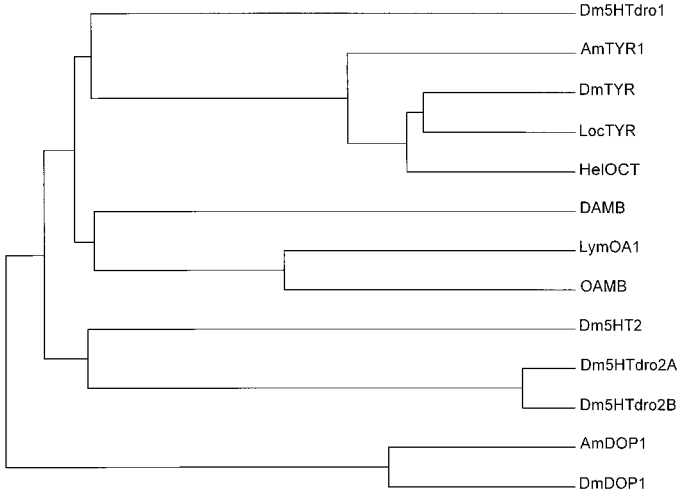

FIG. 2. A: Amino acid comparison of AmTYR1 and cloned tyramine receptors from *Drosophila* [DmTYR (Arakawa et al., 1990; Saudou et al., 1990)] and *Locusta* (Vanden Broeck et al., 1995) and an octopamine receptor from *H. virescens* (von Nickisch-Rosenegk et al., 1996). Putative TM regions (TM1–TM7) are overlined. The amino acid position is indicated at the right. Residues identical to those of AmTYR1 are given as white letters against black; conservative substitutions are shaded. Dashes indicate gaps that were introduced to maximize homologies. Numbers in parentheses correspond to the number of amino acids that are not shown. **B:** Dendrogram of AmTYR1 and cloned invertebrate biogenic amine receptors. Sequence alignment was done with the CLUSTAL program of PCGENE software (version 6.6; IntelliGenetic), using the complete amino acid sequence of each receptor. The honeybee tyramine receptor (AmTYR1) was aligned with tyramine receptors from *Drosophila* and *Locusta* (DmTYR, accession no. P22270; LocTYR, no. X69520), octopamine receptors from *Drosophila* (OAMB, no. AAC17442), *H. virescens* (HelOCT, no. X95606), and *Lymnea stagnalis* (LymOA1, no. U62771), dopamine receptors from *Drosophila* and the honeybee (DmDOP1, no. X77234; DAMB, no. U61264; and AmDOP1, no. Y13429), and serotonin receptors from *Drosophila* (Dm5HTdro1, no. P20905; Dm5HTdro2A/2B, nos. P28285/P28286; and Dm5HT2, no. X81835).

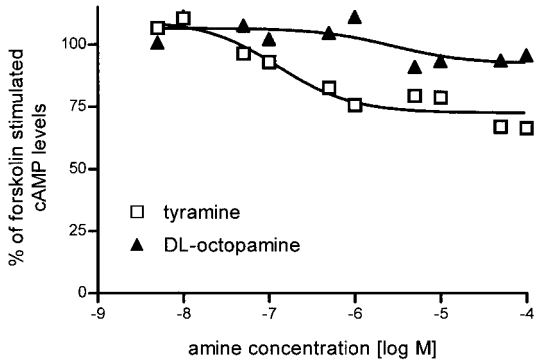

FIG. 3. Tyramine-induced decrease in cAMP levels in cells expressing the AmTYR1 receptor. The cAMP concentration is expressed as the percentage of the value obtained with 20 μM forskolin (defined as 100%). The effect of various concentrations of either tyramine or DL-octopamine on forskolin-stimulated cAMP levels is shown. Data are mean values of at least three independent experiments conducted in duplicate.

less pronounced (~10% decrease of cAMP concentration). Because we used membrane fractions from honeybee brains in these assays, it is possible that tyramine binds to different, probably octopamine receptors that rather activate than inhibit adenylyl cyclase. We therefore tested whether octopamine receptors were present in the membrane fractions used. In assays that were performed in the presence of forskolin and octopamine, we observed a stimulation of cAMP synthesis (Fig. 4). Therefore, it seems reasonable to assume that tyramine at high concentrations ($\geq$10 μM) binds to both tyramine receptors that inhibit adenylyl cyclase and octopamine receptors that activate the enzyme. The promiscuous coactivation of octopamine receptors subsequently counteracts the reduction of cAMP synthesis induced by tyramine receptors.

Expression pattern of the Am*tyr*1 gene

We examined the distribution of Am*tyr*1 mRNA by in situ hybridization to cryosections of the honeybee brain. Labeling of cell somata was observed in most of the brain neurons, including neurons of the optic lobes, mushroom body intrinsic neurons, and neurons of the deutocerebrum (Fig. 5). The abundance of hybridization signals agrees well with our screening data that suggested a relatively high expression level of the Am*tyr*1 gene based on the detection of many positive clones in the cDNA library. It is interesting that the distribution of Am*tyr*1 mRNA closely resembles the pattern that was recently described for the mRNA of a dopamine D1 receptor (Blenau et al., 1998).

DISCUSSION

In this report we describe the molecular and functional properties of a biogenic amine receptor from *A. mellifera.* Phylogenetic analysis of the deduced amino acid sequence showed that the AmTYR1 receptor belongs to the subfamily of invertebrate tyramine receptors. Activation of the receptor by tyramine leads to the inhibition of cAMP production. The gene is abundantly expressed in many areas of the honeybee brain, suggesting that AmTYR1 participates in neural pathways that are involved in the processing of different sensory modalities and possibly higher-order information processing.

Structural properties of the tyramine receptor of *A. mellifera*

The AmTYR1 receptor belongs to the superfamily of G protein-coupled receptors (Donelly et al., 1994). In addition to the presence of seven potential membrane spanning segments, most of the highly invariant residues that are characteristic of biogenic amine receptors (Baldwin et al., 1997) are conserved in AmTYR1. Tyramine and octopamine are structurally similar to norepinephrine, and the binding properties of adrenergic ligands to vertebrate receptors have been thoroughly studied. Several amino acid residues located within the TM regions of the receptor proteins were identified that participate in binding to the ligands (Strader et al., 1989). A pair of regularly spaced serine residues in TM5 was shown to interact with hydroxyl groups of the catechol moiety. Substitution of these serine residues by aliphatic amino acids led to a large decrease in binding affinity of the receptor (Strader et al., 1989). The AmTYR1 receptor also possesses this signature sequence (S_{200}SLGS; see Fig. 1A), suggesting that the hydroxyl group of the benzoyl ring of tyramine can form hydrogen bonds with one of these serine residues, which has not yet been identified.

The signal transmission from G protein-coupled receptors to intracellular effector systems is achieved by binding to specific G proteins. The specificity of this physical interaction is determined, at least in part, by stretches of amino acid residues located in the third intracellular loop as well as in the C terminus of the receptor (Bourne, 1997; Wess, 1997). Cysteine residues

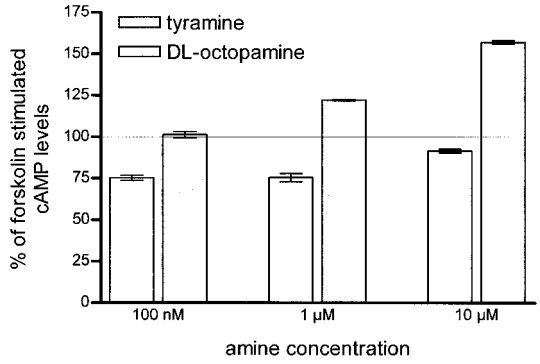

FIG. 4. Effect of tyramine and DL-octopamine on cAMP production in membrane preparations of honeybee brain. The de novo synthesis of [^{32}P]cAMP was measured after incubation of membrane homogenates from honeybee brains with 10 μM forskolin (defined as 100%) and different concentrations of either tyramine or DL-octopamine. Data are mean values of two independent experiments performed in duplicate. Error bars indicate minimal and maximal values.

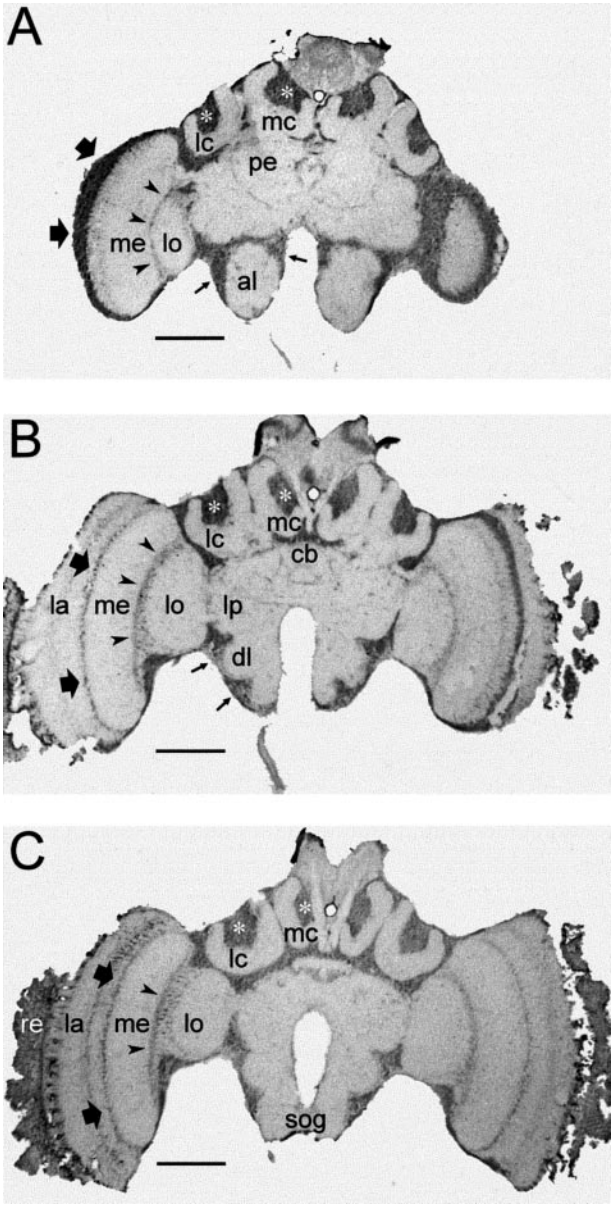

FIG. 5. In situ hybridization of Am*tyr*1 antisense riboprobes to tissue sections of the honeybee brain. Frontal sections (dorsal is to the top) of the anterior (**A**), central (**B**), and posterior (**C**) regions of the honeybee brain are shown. Specific labeling is seen in the somata of most of the brain neuropils, especially in somata of mushroom body intrinsic neurons (asterisks) and in somata surrounding the antennal and dorsal lobes (arrows). Somata within the first (bold arrows) and second (arrowheads) optic chiasmata are also labeled. No specific staining was observed with a sense probe (data not shown). al, antennal lobe; cb, central body; dl, dorsal lobe; la, lamina; lc, lateral calyx of the mushroom body; lo, lobula; lp, lateral protocerebral neuropil; me, medulla; mc, median calyx of the mushroom body; pe, pedunculus of the mushroom body; re, retina; sog, subesophageal ganglion. Bar = 300 μm.

in the C terminus of different biogenic amine receptors were found to undergo posttranslational palmitoylation (O'Dowd et al., 1989). This modification generates a fourth intracellular loop that also participates in receptor–G protein binding (O'Dowd et al., 1989). Because a cysteine is missing in the C terminus of AmTYR1, the fourth intracellular loop does not exist. However, the absence of this domain and the fact that the third intracellular loop of AmTYR1 is shorter than that of other members of this receptor family (see Fig. 2A) do not impair functional coupling of the honeybee receptor. Activation of AmTYR1 in stably transfected HEK 293 cells leads to an inhibition of forskolin-induced cAMP production. This result suggests that AmTYR1 activates endogenous G_i proteins of the HEK 293 cells. Biogenic amine receptors that inhibit adenylyl cyclase activity often possess short C termini (Probst et al., 1992). This structural feature is conserved in AmTYR1 and in tyramine receptors cloned from *Drosophila* (Arakawa et al., 1990; Saudou et al., 1990) and *L. migratoria* (Vanden Broeck et al., 1995), as well as in an octopamine receptor recently cloned from *H. virescens* (von Nickisch-Rosenegk et al., 1996). When heterologously expressed, all receptors inhibit adenylyl cyclase. In addition to the short C terminus, these invertebrate receptors possess highly conserved amino acid sequences. It is notable that almost identical amino acid sequences exist in the vicinity of TM5 and TM6 within the third intracellular loop (see Fig. 2A). Because all four receptors couple to the same intracellular signaling pathway, it is most likely that these residues participate in receptor–G protein coupling.

However, stimulation of the *Drosophila* DmTYR receptor expressed in either Chinese hamster ovary cells (Robb et al., 1994) or *Xenopus* oocytes (Reale et al., 1997) was found to increase $[Ca^{2+}]_i$. We did not observe an AmTYR1-mediated increase in $[Ca^{2+}]_i$ in HEK 293 cells with any biogenic amine tested. These results suggest that agonist-specific coupling of a receptor to different second messenger systems depends on the specific supply of G proteins and might vary between cell types used for heterologous expression studies (Reale et al., 1997).

Functional implications of the AmTYR1 receptor

Although the role of octopamine as a neuroactive substance is well established, a physiological function of tyramine is still elusive in invertebrates. The distribution of both biogenic amines, however, was studied in different invertebrate species (David and Coulon, 1985). Tyramine is abundantly synthesized in the CNS of arthropods, and the octopamine to tyramine ratio varies in different regions of the CNS (Maxwell et al., 1978; Juorio and Sloley, 1988; Downer et al., 1993). Because octopamine is biochemically synthesized from tyramine by hydroxylation of the C_β position, the observed differences in the distribution of these amines might be due to a tissue-specific difference in the kinetics of the tyramine turnover rate. This explanation could support the notion suggesting that tyramine is merely the precursor of octopamine without having any significant physiological function itself.

Both octopamine and tyramine modulate behavioral responses such as the proboscis extension reflex in the honeybee in a similar manner (Braun and Bicker, 1992). Besides evoking similar effects, specific reactions to either amine have been described as well. When either octopamine or tyramine was applied at 100 μM to *Drosophila* head homogenates, an increase in adenylyl cyclase activity was observed (Uzzan and Dudai, 1982). Because octopamine receptors are known to activate adenylyl cyclase, it was assumed that tyramine at high concentrations acts as an octopamine receptor agonist (Uzzan and Dudai, 1982). In coincubation experiments with 20 μM octopamine and 100 μM tyramine, however, tyramine attenuated the octopamine-induced stimulation by 50% (Uzzan and Dudai, 1982). It was assumed that tyramine activates specific receptors that inhibit adenylyl cyclase in *Drosophila* head homogenates, thereby reducing the stimulatory effect of octopamine.

The cAMP system plays a critical role in olfactory learning in the honeybee and *Drosophila* (Davis et al., 1995; Hildebrandt and Müller, 1995; Meller and Davis, 1996; Menzel and Müller, 1996). It is assumed that the action of reinforcers received during training is mediated by aminergic, probably octopaminergic and/or dopaminergic, modulatory neurons that activate G protein-coupled receptors on intrinsic mushroom body neurons (Davis, 1996; Han et al., 1996; Menzel and Müller, 1996; Hammer, 1997; Han et al., 1998). The activated receptors lead to an increase in $[cAMP]_i$, which is supposed to modulate the synaptic output of mushroom body neurons. However, analysis of the *Drosophila* learning mutants *dunce* (*dnc*, encodes a cAMP phosphodiesterase) and *rutabaga* (*rut*, encodes an adenylyl cyclase) as well as *dnc*/*rut* double mutants has shown that the precise modulation of $[cAMP]_i$ is more important to neuronal plasticity than the absolute amount of $[cAMP]_i$ (Tully and Quinn, 1985; Meller and Davis, 1996). In this respect, tyramine and the AmTYR1 receptor could fulfill an important regulatory function because their action potently diminishes an increase of $[cAMP]_i$ evoked by any other modulator. In addition, the Am*tyr*1 gene is expressed in the antennal lobes and the mushroom bodies, two brain regions that are essential for olfactory learning (Erber et al., 1980; Davis, 1993; Meller and Davis, 1996; Menzel and Müller, 1996). Therefore, the AmTYR1 receptor is a suitable candidate for participating in behavioral plasticity of the olfactory system of insects. However, it will be necessary to evaluate the effects of tyramine and specific AmTYR1 receptor ligands in behavioral pharmacological experiments, which can be conducted with the honeybee under laboratory conditions (Blenau and Erber, 1998). The combination of molecular and behavioral approaches should lead to a further understanding of the function of the tyraminergic system in the honeybee.

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Developmental Expression of a Tyramine Receptor Gene in the Brain of the Honey Bee, *Apis mellifera*

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ABSTRACT

This study reveals that the tyramine receptor gene, *Amtyr1*, is expressed in the developing brain, as well as in the brain of the adult worker honey bee. Changes in levels of *Amtyr1* expression were examined using Northern analysis. Age-related increases in *Amtyr1* transcript levels were observed not only during metamorphic adult development, but also in the brain of the adult worker bee. RNA in situ hybridization revealed the pattern of *Amtyr1* expression. Cell bodies staining intensely for tyramine receptor-gene transcript were observed throughout the somata rind, with well-defined clusters of cells associated with developing mushroom bodies, optic lobes, and antennal lobes of the brain. Staining for *Amtyr1* transcript was particularly intense within the three major divisions of mushroom body intrinsic neurons (outer compact, noncompact, and inner compact cells), suggesting that *Amtyr1* is highly expressed in these structures. Activation of *Am*TYR1 receptors heterologously expressed in insect (*Spodoptera frugiperda*) cells led to a reduction in intracellular levels of cAMP similar to that reported for *Am*TYR1 receptors expressed in mammalian (HEK 293) cells (Blenau et al. [2000] J Neurochem 74:900–908). Taken together, these results suggest that *Am*TYR1 receptors may play a role in the developing brain as well as in the brain of the adult worker bee. The actions of tyramine are likely to be mediated, at least in part, via the cAMP-signaling pathway. J. Comp. Neurol. 483: 66–75, 2005.

Indexing terms: biogenic amine; neuromodulation; metamorphosis; invertebrate

Tyramine for many years was viewed solely as the direct precursor amine for the biosynthesis of the neuromodulator octopamine, but there is now compelling evidence that tyramine itself plays a neuroactive role in invertebrate nervous systems (Downer et al., 1993; Kutsukake et al., 2000; Nagaya et al., 2002; Donini and Lange, 2004; recently reviewed by Blenau and Baumann, 2003). The release of this phenolamine from vesicular storage sites and reuptake of synaptically released tyramine via tyramine-specific mechanisms has been demonstrated (Downer et al., 1993) and in a number of invertebrate species, tyramine-specific receptors have been identified (Saudou et al., 1990; Arakawa et al., 1990; Hirip et al., 1994; Vanden Broeck et al., 1995; Blenau et al., 2000; Rex and Komuniecki, 2002; Ohta et al., 2003).

Tyramine receptors are G-protein-coupled receptors (GPCRs), the activation of which leads to changes in second messengers, such as cyclic nucleotides, phosphoinositides, or Ca^{2+} (reviewed by Blenau and Baumann, 2001; Baumann et al., 2003). Heterologously expressed tyramine receptor genes from fruit fly (*Tyr-dro*; Saudou et al., 1990; Robb et al., 1994; Chatwin et al., 2003), locust (*Tyr-Loc*; Vanden Broeck et al., 1995), honey bee (*Amtyr1*;

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Blenau et al., 2000), silkworm (*B96Bom*; Ohta et al., 2003), and *Caenorhabditis elegans* (ser-2; Rex and Komuniecki, 2002) all lead to a reduction of intracellular levels of cAMP in the presence of tyramine. Interestingly, stimulation of the *Drosophila* receptor or the locust receptor not only reduces intracellular cAMP, but also leads to increased levels of intracellular Ca^{2+} (Robb et al., 1994; Poels et al., 2001), indicating that these receptors may be coupled to more than one second messenger pathway. However, this property may not be shared by all insect tyramine receptors. Activation of the honey bee tyramine receptor *Am*TYR1 expressed in HEK 293 cells, for example, has no effect on intracellular Ca^{2+} levels in these cells (Blenau et al., 2000).

Tyramine receptor transcripts can be detected at early stages of insect development (Vanden Broeck et al., 1995; Hannan and Hall, 1996; Ohta et al., 2003) and repeated injection of tyramine into locust larvae is reported to delay ecdysis and reduce locust viability (Torfs et al., 2000). In the fruit fly, levels of tyramine receptor expression fluctuate during embryonic, larval, and pupal development (Hannan and Hall, 1996), and in the silkworm developmental changes in tyramine levels have also been identified (Hirashima et al., 1999). The honey bee tyramine receptor gene, *Amtyr1*, is widely expressed in the brain of the adult worker bee (Blenau et al., 2000), but whether it is expressed in the developing brain is unknown. Here we focus attention on the prominent and highly structured mushroom bodies (MBs) of the brain.

MBs are higher-order sensory integration centers (Erber, 1978; Li and Strausfeld, 1997, 1999; Strausfeld, 2001) that receive processed sensory input from primary sensory centers of the brain and subesophageal ganglion (Mobbs, 1982; Rybak and Menzel, 1993; Schröter and Malun, 2000; Abel et al., 2001; Gronenberg, 2001; Ehmer and Gronenberg, 2002; Strausfeld, 2002; Schröter and Menzel, 2003). Each adult MB is comprised of a large number of densely packed intrinsic neurons, called Kenyon cells (after Kenyon, 1896), the dendritic arbors of which form cup-shaped calyces of the MBs (see Fig. 1). Approximately 170,000 Kenyon cell bodies reside within the calyces of each MB (Witthöft, 1967) and an estimated 14,000 Kenyon cell bodies lie outside each calycal cup (Strausfeld, 2002). Farris et al. (1999) have shown that subpopulations of MB Kenyon cells are born at different but overlapping developmental periods and that they are derived from four clusters of neuroblasts that are already identifiable in newly hatched honey bee larvae. These centrally placed regions of cell proliferation give rise to concentric layers of Kenyon cells. Outer compact cells (OCCs) are born first and are pushed to the periphery of the calyces by the birth of noncompact cells (NCCs). NCCs in turn are pushed outward by inner compact cells (ICCs), which reside at the center of each calycal cup (Farris et al., 1999; Fig. 1). Kenyon cells within each subpopulation develop highly distinctive morphological profiles and, together, Kenyon cell processes form the MB neuropil (reviewed by Strausfeld, 2002). Stagewise changes in morphology associated with the development of the MBs have been described in detail elsewhere (Malun, 1998; Farris et al., 1999; Ganeshina et al., 2000; Schröter and Malun, 2000). These very beautiful developmental studies provide the framework for our ongoing analysis of neuromodulatory actions of biogenic amines and their roles in the developing brain.

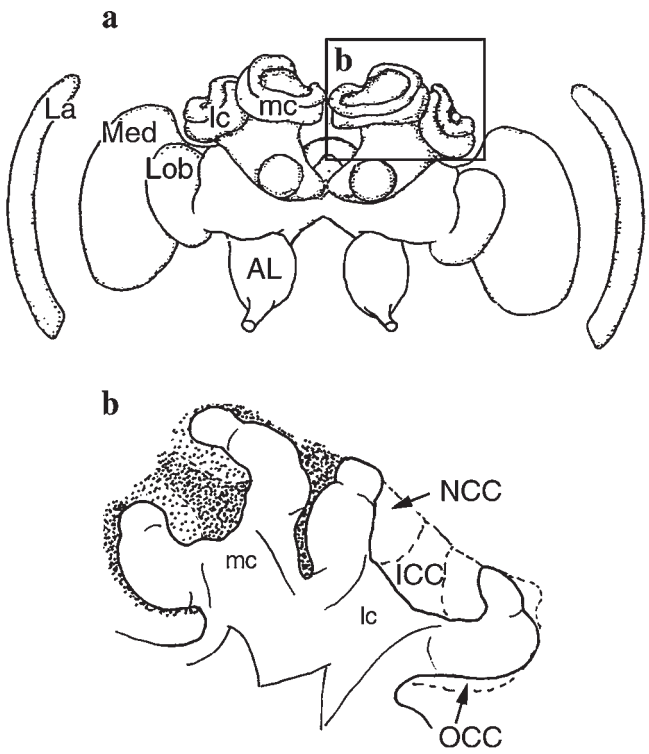

Fig. 1. **a:** Diagrammatic representation of the honey bee brain (modified from Mobbs, 1985). AL, antennal lobe; La, lamina; Med, medulla; Lob, lobula; lc, lateral calyx of MB; mc, medial calyx of MB. **b:** Diagram showing major subpopulations of Kenyon cells lying in and around the lateral (lc) and medial (mc) calyces of the left MB (modified from Kurshan et al., 2003). OCC, outer compact cells; NCC, noncompact cells; ICC, inner compact cells.

In this study, we examine the developmental expression of *Amtyr1*. The study has three goals: 1) to confirm the functional properties of the *Am*TYR1 receptor expressed in an insect cell line; 2) to examine changes in the levels of *Amtyr1* expression during metamorphic adult development of the brain; and 3) to identify cells in the developing brain expressing *Amtyr1* transcript. The study focuses on the expression of *Amtyr1* in developing MB Kenyon cells. However, our results show that this gene is expressed not only in MBs, but also in other prominent brain regions such as antennal lobes and optic lobes of the brain.

MATERIALS AND METHODS

Functional analysis of *Amtyr1*

Activation of *Am*TYR1 receptors heterologously expressed in human embryonic kidney (HEK) cells reduces intracellular levels of cAMP (Blenau et al., 2000). We examined whether *Am*TYR1 receptors show the same property if expressed instead in an insect (*Spodoptera frugiperda*, Sf9) cell line. In addition, we compared the effects on intracellular cAMP elicited by activation of honey bee *Am*TYR1 receptors with responses resulting from activation of the *Drosophila* tyramine receptor, TYR-Dro (Saudou et al., 1990). A baculovirus expression system was used to transiently express *Am*TYR1 receptors or TYR-Dro receptors in Sf9 cells.

Construction of baculovirus recombinants

Recombinant viruses containing either *Amtyr1* or *Tyr-dro* were made using the Bac-To-Bac Baculovirus expression system (Invitrogen, La Jolla, CA). A 2,070 bp *Eco*RI fragment containing the coding region plus ~110 bp 5′ UTR and 760 bp 3′ UTR of the *Amtyr1* cDNA (Blenau et al., 2000) or a 3315 bp *Eco*RI fragment containing the coding region plus 317 bp 5′ UTR and 1193 bp 3′ UTR of the *Tyr-dro* cDNA (Saudou et al., 1990) was ligated into *Eco*RI-digested pFastBac. The orientation of the insert was confirmed so that *Amtyr1* or *Tyr-dro* would be expressed under the control of the polyhedrin promoter. The pFastBac-receptor gene shuttle vectors were then transformed into *E. coli* (strain DH10Bac) containing the baculovirus genome bacmid (bMON14272). The region containing the polyhedrin promoter and the *Amtyr1* or *Tyr-dro* cDNA was recombined from the shuttle vector to the bacmid using Tn7 transposase. The recombinant bacmid was then purified and transfected into Sf9 cells using cellfectin (Invitrogen). Virus stocks were produced using protocols described in the Bac-To-Bac manual.

Determination of intracellular levels of cAMP

Exponentially growing Sf9 insect cells were diluted into serum-free medium (Sf-900 II SFM, Invitrogen). Cells (1.8×10^5/well) were transferred into 24-well culture dishes and allowed to adhere overnight at 28°C before being infected with the *Amtyr1*-recombinant virus or *Tyr-dro*-recombinant virus at a multiplicity of infection of five. After incubating infected cells for 28 hours in serum-free medium, this medium was removed and replaced with fresh medium containing 10^{-4} M of the cAMP phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX), 10^{-5} M forskolin to stimulate adenylyl cyclase activity, and tyramine at concentrations indicated. Cells were exposed to the drugs for 20 minutes at 28°C. The medium bathing the cells was then removed, the cells were lysed with lysis buffer supplied in the cAMP assay kit, and the amount of intracellular cAMP was determined using a cAMP enzyme immunoassay (Amersham Pharmacia Biotech, Piscataway, NJ). Each sample was analyzed in duplicate. A minimum of three independent assays was carried out for each compound. Tyramine hydrochloride, 3-isobutyl-1-methylxanthine (IBMX), and forskolin were purchased from Sigma (St. Louis, MO). Data were analyzed and displayed using Prism 3.0a for Macintosh (GraphPad Software, San Diego, CA).

Analysis of *Amtyr1* expression during development

Nine distinct stages (pupal stages 1 to 9; P1–P9) can be identified during metamorphic adult development of the honey bee. In this study, pupal bees were assigned to one of the nine stages of development by using well-defined characteristics such as eye color and head pigmentation (Jay, 1962; Ganeshina et al., 2000). *Amtyr1* expression levels were also examined in newly emerged adult worker bees and in pollen foragers. Newly emerged adults (NE) were collected as they emerged from their brood cell and pollen foragers (PF), which are generally around 3 weeks of age or older (Winston, 1987), were collected at the hive entrance and identified by the loads of pollen attached to their legs.

Northern analysis

Northern analysis was used to examine changes in levels of *Amtyr1* expression during metamorphic adult development of the brain. The brains of adult worker bees, and of bees at different stages of metamorphic adult development, were dissected from the head capsule, quick frozen in liquid nitrogen, and stored at −80°C. Total RNA was isolated from pupal bee brains and from brains of pollen foragers using TRIZOL LS Reagent (Invitrogen) as described by the manufacturer. Poly $(A)^+$ RNA was isolated from total RNA preparations by using the Micro Fast-Track 2.0 Kit (Invitrogen) as described by the manufacturer. Northern analysis was carried out as described by Sambrook et al. (1989), with minor modifications. Poly $(A)^+$ RNA (~3 μg) was loaded onto a 1% agarose MOPS-formaldehyde gel and fractionated by electrophoresis. For assessment of RNA loading, the gel was stained with 5 μg/ml ethidium bromide in 1 × MOPS buffer, destained in several changes of distilled water, and photographed. RNA was transferred onto positively charged nylon membranes (Roche, Nutley, NJ) overnight by capillary action using 20 × SSC as transfer buffer. RNA was fixed to the membrane by baking at 120°8C for 30 minutes. Probe for *Amtyr1* was labeled with ^{32}P using a nick translation kit (Roche) using a plasmid containing the entire coding region of *Amtyr1* (Blenau et al., 2000) as the template. Hybridization was in 50% formamide, 5 × SSC, 5 × Denhardt's, 0.1 mg/ml denatured salmon sperm DNA, and 0.1% SDS at 42°C for 16 hours. Blots were washed in 1 × SSC, 0.1% SDS for 5 minutes at room temperature and then twice at 60°C for 30 minutes. The membrane was then exposed to X-ray film for 12–72 hours at −80°C. For quantitation of ethidium bromide-stained gels and Northern blots, NIH Image was used. Amounts of *Amtyr1* mRNA relative to the total amount of mRNA loaded were calculated for two independent blots. For presentation purposes, the level of *Amtyr1* mRNA at pupal stage 2 (P2) was set to 1 and the amounts of transcript detected at other stages expressed relative to P2 (see Fig. 3).

Identification of cells expressing *Amtyr1* mRNA

In situ hybridization was used to identify cells in the brain expressing *Amtyr1*. The techniques used for in situ hybridization have been described in detail elsewhere (Kurshan et al., 2003). The brains of bees at all nine stages of metamorphic adult development were examined in this study (P1–P9). The distribution of *Amtyr1*-expressing cells was also examined in newly emerged adults (NE) and in pollen foragers (PF).

Preparation of brain tissue

For pupal bees at stages P1–P5, entire heads were placed in fixative. In the case of adults and pupal bees 6 days of age or older, the brain was dissected from the head capsule under cold PBS (2 mM NaH_2PO_4, 5.8 mM Na_2HPO_4, 154 mM NaCl, pH 7.2). Isolated brains or whole heads were fixed in 4% paraformaldehyde in PBS at room temperature for 2 hours (brains) or 4 hours (heads). The tissue was then transferred to 18% sucrose/PBS for cryoprotection overnight. The following day, the tissue was embedded in freezing medium (Cryomatrix, Shandon, Pittsburgh, PA) and sectioned in a frontal plane into

16-μm slices, which were mounted onto polylysine-coated slides (Sigma).

Probe synthesis

Antisense and sense digoxigenin (DIG)-labeled riboprobes were transcribed from a plasmid containing either a 795 bp *Eco*RI fragment or a 930 bp *Eco*RV fragment from the 3′ untranslated region of the *Amtyr1* cDNA (Blenau et al., 2000) in pBluescript II (Stratagene, La Jolla, CA). Antisense probe was synthesized using T7 RNA polymerase with *Xho*I linearized plasmid, whereas sense probe was synthesized using T3 RNA polymerase with *Xba*I linearized plasmid. DIG-labeled riboprobes were synthesized using a DIG RNA labeling kit (Roche) according to the manufacturer's instructions. For the *Eco*RI probe the number of brains analyzed was: P1, n = 2; P2, n = 2; P3, n = 3; P4 n = 3; P5: n = 2; P6, n = 1; P7, n = 1; P8, n = 1; P9, n = 1; NE, n = 2; PF, n = 7. For the *Eco*RV probe P4, n = 1, PF, n = 2. Both antisense probes gave similar staining patterns (data not shown); the results presented here used the *Eco*RI fragment probe.

In situ hybridization

Tissue sections were treated according to Braissant and Wahli (1998), with minor modifications. Frozen sections of brain tissue were allowed to air-dry for 5 minutes and then immediately postfixed in 4% paraformaldehyde for 20 minutes, followed by inactivation of RNases with two 15-minute washes in PBS containing 0.1% active diethylpyrocarbonate (DEPC; Sigma). Slides were then incubated at 40°C for 10 minutes in 5 μg/ml Proteinase K (Roche) in Tris/EDTA (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) to improve probe penetration, and then equilibrated in 5 × SSC (saline sodium citrate: NaCl 0.75 M, Na-Citrate 0.075 M, pH 7) for 15 minutes. Sections were incubated for 2 hours at 58°C in prehybridization solution (50% formamide, 5 × SSC, 100 μg/ml salmon sperm DNA (Gibco BRL, Gaithersburg, MD; denatured for 5 minutes at 80°C). To this, 0.5 μg/ml of (denatured) DIG-labeled RNA probe was added for hybridization at 58°C for 35–40 hours. Both prehybridization and hybridization reactions were carried out by drawing a hydrophobic barrier around the sections with a Pap Pen (Sigma), adding ~60 μl of solution to each slide and covering it with parafilm (American National Can). Following hybridization, slides were washed in 2 × SSC at room temperature for 30 minutes, and then at 65°C for an hour, followed by another wash in 0.1 × SSC at 65°C for an hour. They were then equilibrated in Buffer 1 (Tris/HCl 100 mM and NaCl 150 mM, pH 7.5) for 5 minutes. Sections were then incubated with alkaline phosphatase-labeled anti-DIG antibody (Roche) diluted 1:5,000 in Buffer 2 (Buffer 1 containing 0.5% blocking reagent; Roche) for 2 hours at room temperature. Excess antibody was removed by two 15-minute washes in Buffer 1, with gentle agitation, and was followed by equilibration in Buffer 3 (Tris/HCl 100 mM, NaCl 100 mM, and $MgCl_2$ 50 mM, pH 9.5) for 5 minutes. The color reaction was performed overnight at room temperature in Buffer 3 containing 4.5 μl/ml nitroblue tetrazolium chloride (NBT; Roche) and 3.5 μl/ml 5-bromo-4-chloro-3-indolylphosphate, 4-toluidine salt (BCIP; Roche). Staining was stopped by a 15-minute wash in Tris/EDTA, and precipitated Tris was removed with two 15-minute washes in deionized water. Any nonspecific staining was removed with a 30-minute wash in 95% EtOH with gentle agitation. Sections were photographed with Fujichrome tungsten slide film (ASA 64) using an Olympus C-35AD-4 camera. The photographs (slides) were scanned (using AGFA Photolook) and saved as PhotoShop files (Adobe Systems, San Jose, CA). Minor contrast and brightness adjustments were made to render the images more clearly. Sense controls were examined for each developmental stage (see Figs. 4–6 for representative examples).

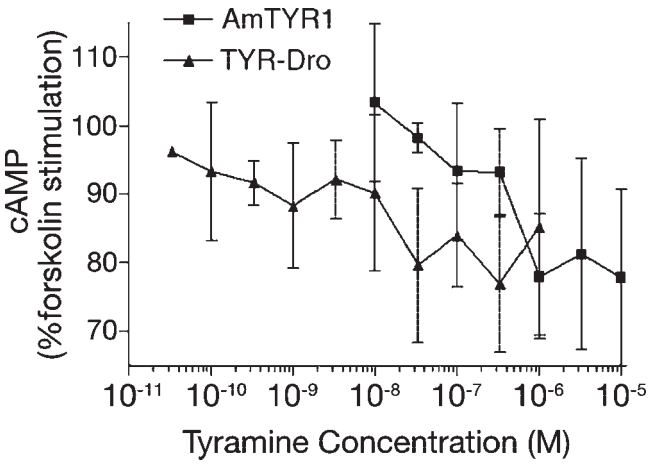

Fig. 2. Effects of tyramine on intracellular cAMP levels of *Spodoptera frugiperda* (Sf9) cells expressing the *Am*TYR1 or TYR-Dro receptor. Cells expressing *Am*TYR1 or TYR-Dro receptors were treated with IBMX, forskolin, and a range of tyramine concentrations, and their intracellular cAMP levels assayed. The cAMP level for cells treated with IBMX and forskolin was taken as 100%. The data shown are the mean ± SEM for four to five independent assays done in duplicate.

RESULTS

Functional properties of *Am*TYR1 receptors expressed in SF9 cells

In forskolin-stimulated Sf9 cells expressing *Am*TYR1 receptors, intracellular cAMP levels decreased in the presence of tyramine. A comparison of responses to tyramine in Sf9 cells expressing *Am*TYR1 or TYR-Dro receptors shows that in both cases the effects of tyramine on intracellular cAMP levels are dose-dependent and activation of the receptors leads to a similar maximum level of cAMP reduction (Fig. 2). The concentration of tyramine required to achieve maximal reduction of cAMP levels was slightly lower in cells expressing TYR-Dro receptors than in cells expressing *Am*TYR1. Half-maximal reduction of cAMP levels (EC_{50}) was observed with ~8.6×10^{-8} M tyramine in *Am*TYR1-expressing cells (pEC_{50} = 7.07 ± 0.45 mean ± SEM, n = 5) and ~1.06×10^{-8} M tyramine in TYR-Dro-expressing cells (pEC_{50} = 7.97 ± 0.66, n = 4).

Changes in *Amtyr1* expression levels associated with brain development

The relative levels of *Amtyr1* expression in the developing honey bee brain were determined using Northern analysis (Fig. 3). When the entire *Amtyr1* coding sequence was labeled and hybridized to honey bee mRNA, a single 9.5 kb band was observed (Fig. 3b). The ratio of *Amtyr1* mRNA to total mRNA loaded was calculated for two inde-

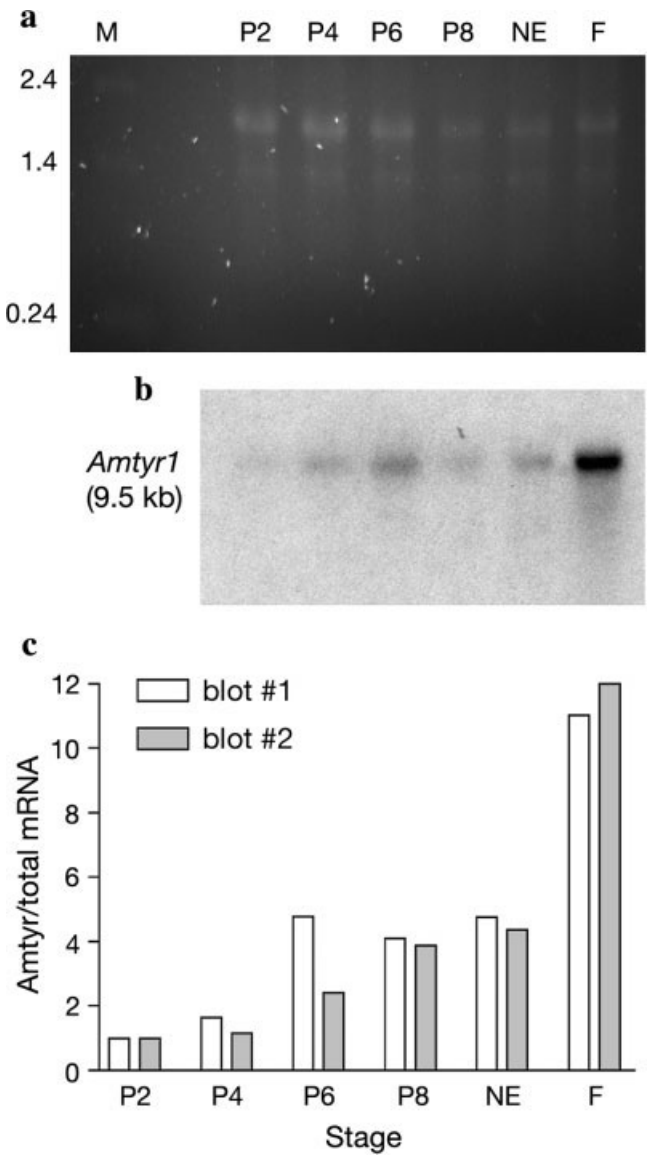

Fig. 3. Northern analysis of *Amtyr1* expression levels during development of the honey bee brain. Two independent Northern blots were analyzed. **a:** Representative ethidium bromide stained gel before transfer showing molecular weight markers in kb (M) and mRNA isolated from pupal (P2, P4, P6, P8) or adult (newly emerged adult, NE; forager, F) brains. **b:** Membrane (blot 1) corresponding to the gel shown in (a) was probed with *Amtyr1* resulting in a single band of ~9.5 kb. **c:** Bar graph illustrating the amount of *Amtyr1* mRNA relative to the amount of total mRNA loaded for two independent Northern blot experiments. To aid comparison, *Amtyr1* mRNA levels at each stage are normalized to the level detected in stage 2 pupae (P2).

pendent blots. To aid comparison, *Amtyr1* mRNA levels in all groups are normalized to levels detected at pupal stage 2 (Fig. 3c). *Amtyr1* transcript levels increase ~4-fold during metamorphic adult development (Fig. 3c; P2–P8). Interestingly, transcript levels increase still further after adult eclosion, with levels of *Amtyr1* transcript in pollen foragers ~3-fold higher than the levels detected in the brains of newly emerged adults (Fig. 3c, NE, F).

Expression of *Amtyr1* by neurons in the developing brain

To examine the distribution of cells in the brain expressing *Amtyr1*, in situ hybridization analysis was undertaken on brain sections from pupae at different stages of development, as well as on newly emerged adults and pollen foragers. This component of the study begins at the first pupal stage (P1) when outer compact cells (OCCs) have already been born, the birth of noncompact cells (NCCs) is under way, the earliest arising components of the vertical and medial lobes of the MBs have already been laid down, and the calycal neuropil has just begun to form (Farris et al., 1999).

In Figures 4–6, representative sections treated with antisense *Amtyr1* probe are presented alongside adjacent sections from the same brains treated with the sense (control) probe. Treatment of brain sections with sense probe produces no labeling, whereas sections treated with antisense probe exhibit strong site-specific staining of cells and cell clusters in the developing brain (Figs. 4, 5), as well as in the brain of the adult worker bee (Fig. 6). Analysis of brain tissue at different stages of development revealed marked changes in the expression pattern of *Amtyr1* associated, in particular, with the development of MB Kenyon cells.

Expression of *Amtyr1* in developing intrinsic MB neurons (Kenyon cells)

At P1 there is intense staining for *Amtyr1* mRNA in the OCCs (Fig. 4ai, arrows), which at this stage lie below and to the sides of each cluster of neuroblasts (Farris et al., 1999). The noncompact cells (NCCs), which have a slightly larger diameter, show little if any staining at P1, but by P2 staining is clearly evident in these cells also (Fig. 4b, arrowheads), although the staining is not yet as intense as that seen in the OCCs. By P3, the reverse is true: staining is now more intense in developing NCCs (Fig. 4c, arrowhead) than in OCCs (Fig. 4c, arrow), but there is little or no staining of inner compact cells (ICCs; Fig. 4c, double-headed arrow), which start being born at P3 (Farris et al., 1999). By P4, staining in the OCCs (Fig. 4d. arrow) is less intense than in NCCs (Fig. 4d, arrowhead) and staining is now apparent in ICCs (Fig. 4d, double-headed arrow). This pattern remains stable throughout the remaining stages (P5–P9) prior to adult eclosion (compare Fig. 5, P5; Fig. 6a, P9). In the adult brain, however, staining intensity in NCCs has fallen and now appears more intense in ICCs than in either the OCCs or NCCs. This adult pattern is already apparent in newly emerged adults (Fig. 6b, arrow), as well as in pollen foragers (Fig. 6cii, double arrowhead). At no stage in the development of the MBs was staining for *Amtyr1* apparent within the cell proliferation zone. This area is shown most clearly in Figure 4d (asterisk).

Expression of *Amtyr1* in protocerebral neurons other than MB neurons, and in deutocerebral and tritocerebral neurons

In addition to the cell populations described above, there was a scattered distribution of protocerebral, deutocerebral, and tritocerebral somata in which staining was as intense as that seen in developing Kenyon cells of the MBs. Some of these intensely staining cells were grouped into clusters and these are described below.

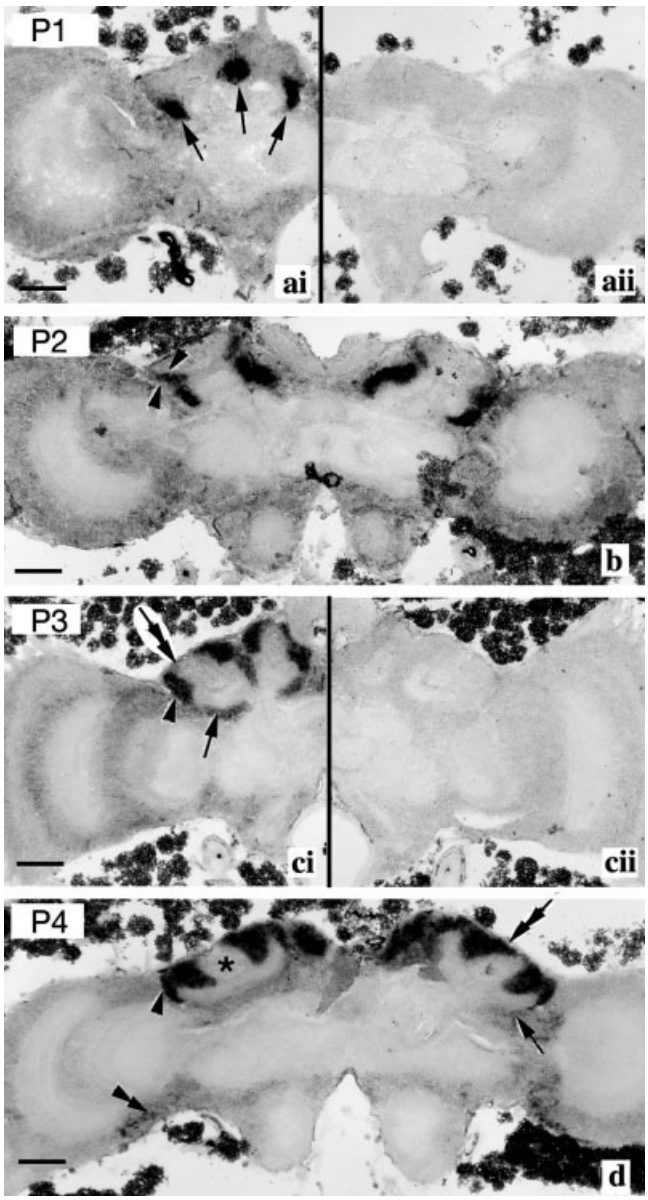

Fig. 4. In situ hybridization analysis of *Amtyr1* expression in the brains of pupal bees at P1–4. **a:** Pupal stage 1. **b:** Pupal stage 2. **c:** Pupal stage 3. **d:** Pupal stage 4. For pupal stages 1 and 3, staining using the antisense probe (**ai, ci**) is compared with staining using the sense (control) probe (**aii, cii**, respectively). Arrows, OCCs. Arrowheads, NCCs. Double-headed arrows, ICCs. Double arrowheads, cell clusters lying along the ventral and dorsal margins of the optic lobes. Asterisk, proliferative region. Scale bars = 200 μm.

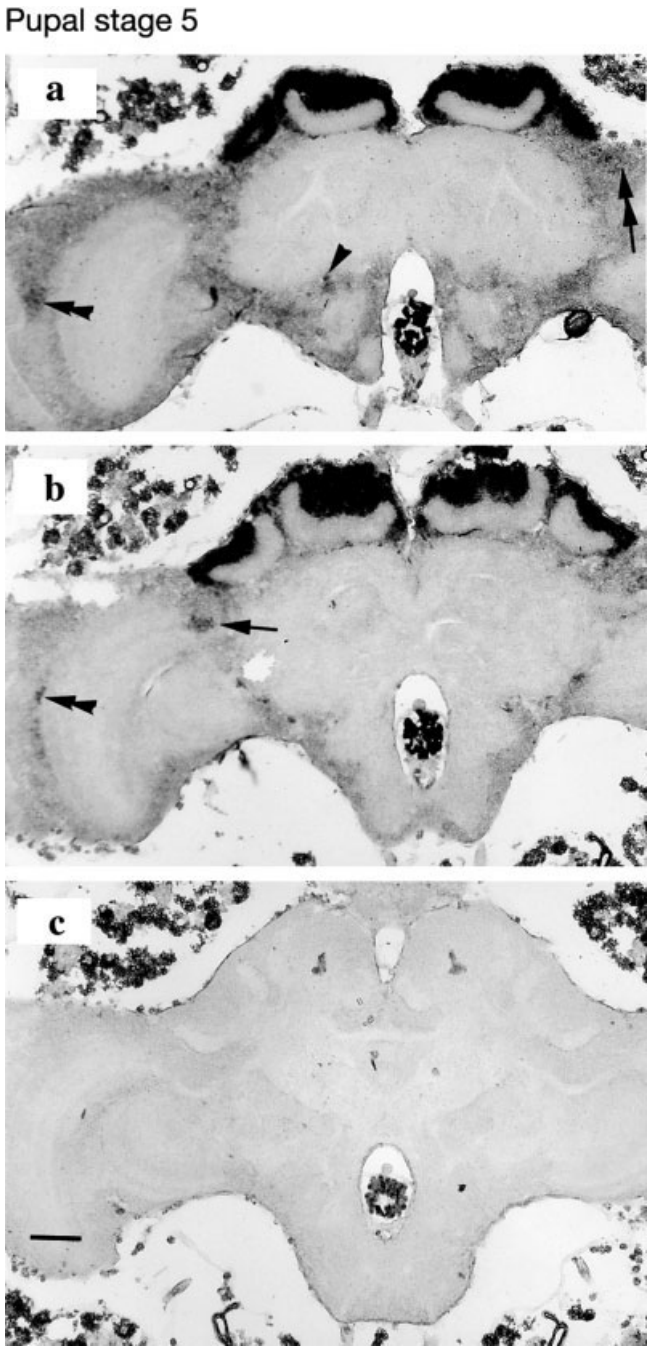

Fig. 5. *Amtyr1* expression in the brain at P5. In addition to the intense staining in mushroom body Kenyon cells, in situ hybridization analysis reveals staining for *Amtyr1* mRNA in other cells and cell clusters of the somatal rind. **a:** Anterior brain section showing diffuse clusters of cells lying along the dorsal (double-headed arrow) margin of the optic lobe, a cluster lying on the anterior margin of the optic lobe between the developing neuropil of the lamina and medulla (double arrowhead), and a small cluster of intensely staining cells on the dorsal margin of the antennal lobe (arrowhead). **b:** Posterior brain section showing a cluster of *Amtyr1*-expressing cells located between the dorsal margin of the medulla and the lateral protocerebral neuropil (arrow), and a band of intensely staining cells between the medulla and lamina of the optic lobe (double arrowhead). **c:** Section from the same brain as (a) and (b) but stained with sense (control) probe. Scale bar = 200 μm.

In both hemispheres of the brain a large cluster of cells lying along the ventral margin of the developing optic lobe neuropil could be identified at all stages of development (Fig. 4d, double arrowhead). A less prominent cell cluster could be identified also along the dorsal margins of each optic lobe (e.g., Fig. 5a, double-headed arrow; see also Fig. 4d). Within these clusters, cells showing intense staining with *Amtyr1* probe were surrounded by cells exhibiting very low-level staining, giving each cluster a more diffuse appearance than the densely packed clusters of intensely

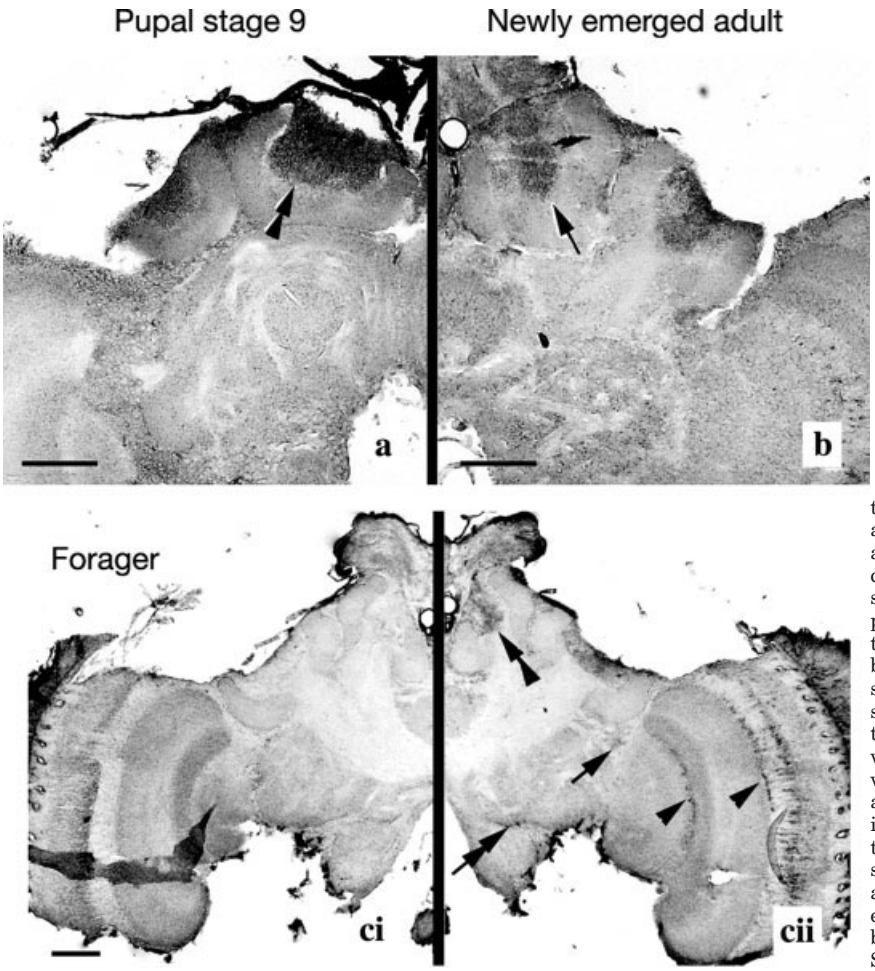

Fig. 6. *Amtyr1* expression in the brain prior to (**a**), immediately after (**b**), and some weeks after (**c**) adult emergence. Staining artifacts are apparent at the margins of some sections (see a, ci, cii). a: A section of the brain at P9 reveals staining of similar intensity in NCCs and ICCs prior to adult ecdysis. Together these cells fill the calycal cup of the MBs (double arrowhead). b: In newly emerged adults, ICCs (arrow) stained more intensely for *Amtyr1* mRNA than surrounding NCCs. c: *Amtyr1*-expressing cells in the brain of a pollen forager. ci: Section stained with sense (control) probe. cii: Section stained with antisense probe. As in newly emerged adults (b), staining for *Amtyr1* mRNA was more intense in ICCs (double arrowhead) than in either the NCCs or OCCs. Clusters of intensely staining cells can also be identified dorsal to the antennal lobe (double-headed arrow), on the lateral margin of the protocerebrum (arrow), and in bands between the optic ganglia (arrowheads). Scale bars = 200 μm.

staining Kenyon cells described above. A discrete cell cluster was identified also on the anterior margin of each optic lobe between the developing lamina and medulla neuropils of the optic lobe (Fig. 5a, double arrowhead), and in deeper layers of the optic lobe discrete bands of cells expressing *Amtyr1* mRNA were observed, one lying between the neuropil of the lobula and medulla (not shown) and a second band located between the neuropil of the medulla and lamina (Fig. 5b, double arrowhead). A further discrete cell cluster staining for *Amtyr1* mRNA was located in an area between the medulla of the optic lobe and lateral protocerebral neuropil (Fig. 5b, arrow). Small groups of intensely staining cells were also detected immediately dorsal to the developing antennal lobe neuropil (Fig. 5a, arrowhead). All of these cell clusters could be identified within the adult brain and most are apparent in Figure 6cii.

DISCUSSION

Our results support four main conclusions. First, the tyramine receptor gene, *Amtyr1*, is expressed in the developing brain as well as in the brain of the adult worker honey bee. Second, *Amtyr1* transcript levels increase during the course of development and show significant variation also in the adult brain. Third, throughout development the strongest staining for *Amtyr1* mRNA is associated with the MBs, suggesting that *Amtyr1* is highly expressed in these structures and may play a role in their construction. Finally, whether expressed in HEK 293 cells (Blenau et al., 2000) or in Sf9 cells (present investigation), activation of *Am*TYR1 receptors leads to a reduction in levels of intracellular cAMP.

*Am*TYR1 receptors and their coupling to the cAMP signaling pathway have been examined previously using transient expression of *Am*TYR1 receptors in human embryonic kidney (HEK 293) cells (Blenau et al., 2000). As the functional properties of a receptor can be affected by the cell line in which the receptor is expressed (see Blenau and Baumann, 2001; Poels et al., 2001), we reexamined the effects of *Am*TYR1-receptor activation on levels of intracellular cAMP using receptors expressed in an insect (Sf9) cell line. As expected, activation of *Am*TYR1 receptors reduced levels of intracellular cAMP. The EC_{50} recorded in this study ($pEC_{50} = 7.07 \pm 0.45$) is similar to that recorded for *Am*TYR1 receptors expressed in HEK cells ($pEC_{50} = 6.89 \pm 0.23$; Blenau et al., 2000). Interestingly, the maximum level of cAMP reduction that can be obtained through the activation of honey bee tyramine receptors expressed in Sf9 cells is remarkably similar to that resulting from activation of the *Drosophila* tyramine receptor, TYR-Dro (Fig. 2). The concentration of tyramine

required to achieve maximal reduction of cAMP levels, however, was slightly lower in cells expressing TYR-Dro receptors than in cells expressing *Am*TYR1. The EC_{50} value for TYR-Dro receptors ($\sim 1.06 \times 10^{-8}$ M) is similar to that reported recently by Chatwin et al. (2003), who expressed TYR-Dro in CHO cells, but the same receptor expressed in mouse NIH 3T3 cells gives a reported EC_{50} of 2.4×10^{-6} M (Saudou et al., 1990). Our results confirm that the actions of tyramine in the brain of the bee are likely to be mediated, at least in part, via the cAMP signaling pathway.

The presence of *Amtyr1* mRNA within discrete brain-cell clusters establishes tyramine as a likely candidate to play a neuroactive role in the brain of the bee. Interestingly, the distribution of *Amtyr1* mRNA in the adult worker bee is similar to that described for the fruit fly (Hannan and Hall, 1996; Kutsukake et al., 2000). Cell bodies that stain intensely for tyramine receptor-gene transcript are scattered throughout the brain, with clusters of cells associated not only with the MBs, but also within the optic lobes and antennal lobes of the brain. The roles that tyramine receptors play in these regions of the insect brain remain unclear, but reports describing the behavior and physiology of *Drosophila* mutants with abnormalities in tyraminergic pathways may provide interesting clues. Adults of the tyramine receptor mutant *honoka* (*hono*) express reduced levels of TYR-dro receptor and exhibit abnormal olfactory behavior, including reduced levels of avoidance in response to odors that are normally repellent (Kutsukake et al., 2000). Mutant larvae that have altered levels of tyramine ($T\beta h^{nM18}$: Monastirioti et al., 1996; *iav*: O'Dell, 1993; McClung and Hirsh, 1999) also exhibit abnormal locomotor behavior (Saraswati et al., 2004). Exogenously applied tyramine also alters the behavior of bees, increasing their responsiveness to sucrose (Scheiner et al., 2002). Some of these effects, however, may reflect peripheral rather than central actions of tyramine, as tyramine has modulatory actions on many peripheral tissues (e.g., Downer, 1979; Huddart and Oldfield, 1982; Nagaya et al., 2002; Blumenthal, 2003; Donini and Lange, 2004).

In the brain, staining for *Amtyr1* transcript was most prominent in MB Kenyon cells, which also express the dopamine receptor genes, *Amdop1* and *Amdop2* (Kurshan et al., 2003). The pattern of *Amtyr1* expression, however, differs from that of either *Amdop1* or *Amdop2*. Staining for *Amdop1* mRNA is particularly intense in newly born Kenyon cells (Kurshan et al., 2003), but this was not the case for *Amtyr1*. At P1, for example, NCCs show strong staining for *Amdop1* mRNA (Kurshan et al., 2003), but little, if any, staining for *Amtyr1* (Fig. 2). At P3, the birth of ICCs begins (Farris et al., 1999). There is intense staining of these cells for *Amdop1* mRNA at this stage (Kurshan et al., 2003), but *Amtyr1* transcript was not detected in these cells until at least one stage later, around P4 or P5 (Figs. 4, 5). Furthermore, *Amtyr1* mRNA was not detected at any stage in layers occupied by glial cells, which early in pupal development express the dopamine receptor gene *Amdop2* (Kurshan et al., 2003). While their detection in the developing brain implicates all three genes in brain development, differences in the spatial and temporal expression of *Amtyr1* (present investigation) and the dopamine receptor genes *Amdop1* and *Amdop2* (Kurshan et al., 2003) suggest that each of these genes plays a different role in the brain of the bee.

Northern analysis revealed significant variation in levels of *Amtyr1* transcript, not only during development, but also in the brain of the adult worker bee. The birth of MB Kenyon cells and high levels of *Amtyr1* expression in these cells during development (Figs. 4, 5) are likely to contribute to the overall increases in *Amtyr1* mRNA detected between P2–8 (Fig. 3). NCCs are still being added at P2, the birth of ICCs begins around P3 and it is not until P7 that the full complement of Kenyon cells is finally in place (Farris et al., 1999). Perhaps more intriguing is the finding that levels of *Amtyr1* transcript as a proportion of total mRNA continue to increase after adult emergence (Fig. 3). This cannot be explained by the birth of new cells, as neurogenesis in the brains of adult honey bees is absent (Fahrbach et al., 1995; Farris et al., 1999; Schröter and Malun, 2000). However, similar age-related changes in receptor gene expression have been reported for the honey bee dopamine receptor gene, *Amdop2* (Humphries et al., 2003), and may reflect alterations in receptor gene expression associated with shifts in the behavior of adult worker bees (see also Whitfield et al., 2003). Young bees generally work inside the hive for the first few weeks of adult life, performing tasks such as cleaning, comb construction, feeding and grooming the queen, or caring for developing larvae. They begin foraging for nectar or pollen usually 2–3 weeks or more after their emergence as adults (Winston, 1987). Humphries et al. (2003) found evidence using in situ hybridization analysis that *Amdop2* expression increases with age, particularly in NCCs of the adult brain. In the present study, however, there were no observed differences in staining intensity for *Amtyr1* mRNA between newly emerged bees and foragers that might explain the differences in *Amtyr1* mRNA levels detected in these two adult groups. In recent work using microarray analysis to compare gene expression in young nurse bees and in foragers, Whitfield et al. (2003) showed that 39% of the genes they examined showed changes in expression levels, with some genes being upregulated and others downregulated. Differences in *Amtyr1* mRNA : total mRNA ratios identified in the present study between newly emerged workers and pollen foragers probably reflect a combination of factors, including the upregulation of *Amtyr1* and the downregulation of other genes.

Amtyr1, Amdop1, and *Amdop2* all code for receptors that are coupled to adenylyl cyclase and all are highly expressed in MBs of the brain (Blenau et al., 1998, 2000; Humphries et al., 2003). cAMP signaling has been strongly implicated in synaptic plasticity and memory formation in insects (reviewed by Davis, 1996; Menzel and Müller, 1996; Dubnau and Tully, 1998; Roman and Davis, 2001) and numerous studies have implicated the MBs in olfactory learning (reviewed by Heisenberg, 1998; Menzel, 2001; Roman and Davis, 2001). Fruit flies that carry *rutabaga, dunce,* or *Pka-CI* (DCO) mutations, for example, are defective for learning. All of these mutations map to genes that are highly expressed in MBs of the brain and all affect proteins involved in the cAMP pathway (reviewed by Roman and Davis, 2001). Similarly, expression of constitutively activated $G\alpha_s$ in the MBs, which increases cAMP levels, disrupts associative learning (Connolly et al., 1996). Their coupling to the cAMP signaling pathway suggests that tyramine receptors could contribute to synaptic plasticity in MBs of the brain. A direct comparison of the TYR-Dro and *Am*TYR1 receptors in this study reveals that while both receptors reduce intracellu-

lar cAMP levels to a similar degree (around 75% of forskolin-stimulated levels), when expressed in Sf9 cells, the fruit fly receptor maximally attenuates cAMP levels at lower tyramine concentrations than the honey bee receptor. The second and third cytoplasmic loops and the C-terminus of GPCRs are involved in the interaction between receptors and G-proteins (Dohlman, 1991; Bourne, 1997; Wess, 1997; Oliveira et al., 1999). The amino acid sequence of the second cytoplasmic loop in *Am*TYR1 and TYR-Dro receptors is identical, and 13 of the 15 residues in the C-terminus are conserved. However, the third cytoplasmic loop is much shorter in the bee receptor (113 amino acids) than in the fruit fly receptor (242 residues). It is hoped that further comparative analysis of these two receptors will provide insight into the relationship between receptor structure and coupling to G-proteins, as well as providing a better understanding of tyramine receptors and their roles in the insect brain.

In summary, our study reveals that the tyramine receptor gene, *Amtyr1*, is expressed in the developing brain as well as in the brain of the adult worker honey bee and that levels of *Amtyr1* transcript vary significantly during the lifetime of this animal. To determine the significance of these results it will be important in future studies to establish the relationship between *Amtyr1* mRNA levels and levels of *Am*TYR1 protein and to demonstrate that *Am*TYR1 receptors are functional not only in adults, but also in the developing brain.

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Der Octopamin-Rezeptor der Honigbiene – AmOA1

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Molecular and functional characterization of an octopamine receptor from honeybee (*Apis mellifera*) brain

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Abstract

Biogenic amines and their receptors regulate and modulate many physiological and behavioural processes in animals. In vertebrates, octopamine is only found in trace amounts and its function as a true neurotransmitter is unclear. In protostomes, however, octopamine can act as neurotransmitter, neuromodulator and neurohormone. In the honeybee, octopamine acts as a neuromodulator and is involved in learning and memory formation. The identification of potential octopamine receptors is decisive for an understanding of the cellular pathways involved in mediating the effects of octopamine. Here we report the cloning and functional characterization of the first octopamine receptor from the honeybee, *Apis mellifera*. The gene was isolated from a brain-specific cDNA library. It encodes a protein most closely related to octopamine receptors from *Drosophila melanogaster* and *Lymnea stagnalis*. Signalling properties of the cloned receptor were studied in transiently transfected human embryonic kidney (HEK) 293 cells. Nanomolar to micromolar concentrations of octopamine induced oscillatory increases in the intracellular Ca^{2+} concentration. In contrast to octopamine, tyramine only elicited Ca^{2+} responses at micromolar concentrations. The gene is abundantly expressed in many somata of the honeybee brain, suggesting that this octopamine receptor is involved in the processing of sensory inputs, antennal motor outputs and higher-order brain functions.

Keywords: biogenic amine, Ca^{2+}, cyclic AMP, G protein-coupled receptor, insect, neuromodulation, tyramine.

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Octopamine is a monophenolic amine that belongs to a group of neuroactive compounds known as biogenic amines. Biochemical and pharmacological experiments suggest that octopamine exerts its effects by binding to membrane proteins that belong to the superfamily of G protein-coupled receptors (GPCRs). These receptor proteins share the structural motif of seven transmembrane (TM) domains (Baldwin *et al*. 1997; Okada *et al*. 2001). Activation of the receptors may lead to changes in the concentration of intracellular second messengers such as cyclic nucleotides [cyclic AMP (cAMP) and cyclic GMP], inositol-1,4,5-trisphosphate and Ca^{2+}. Octopamine-mediated changes in the intracellular concentration of cAMP and/or Ca^{2+} ($[Ca^{2+}]_i$) have been reported for several protostomian species (for reviews, see Roeder 1999; Blenau and Baumann 2001).

Since its discovery in the salivary glands of the octopus (Erspamer and Boretti 1951), octopamine has been found in high concentrations in neuronal and non-neuronal tissues of many nematodes, annelids, arthropods and molluscs (David and Coulon 1985). Owing to its regulatory functions, octopamine is considered to be a neurotransmitter, neuromodulator and/or neurohormone (for reviews, see David and Coulon 1985; Roeder 1999). Many behavioural and physiological reactions have been attributed to the signalling action

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Abbreviations used: $[Ca^{2+}]_i$, intracellular calcium concentration; cAMP, cyclic AMP; ECFP, enhanced cyan fluorescent protein; ES, extracellular solution; GPCR, G protein-coupled receptor; HEK, human embryonic kidney; IBMX, isobutylmethylxanthine; PBS, phosphate-buffered saline; PER, proboscis extension response; PKC, protein kinase C; SDS, sodium dodecyl sulfate; TM, transmembrane; US, unconditioned stimulus.

of octopamine (David and Coulon 1985; Orchard *et al*. 1993), particularly in a number of studies in the honeybee (Braun and Bicker 1992; Erber *et al*. 1993; Burrell and Smith 1995; Pribbenow and Erber 1996; Schulz and Robinson 2001; Scheiner *et al*. 2002). It has been shown that octopamine can modulate the responsiveness of sensory receptors, interneurones and motoneurones, and so affects complex behavioural responses.

Octopamine also plays a major role in olfactory learning and memory formation in the honeybee (Hammer 1993, 1997; Hammer and Menzel 1998; Menzel *et al*. 1999). In these studies, olfactory conditioning of the proboscis extension response (PER) is used as the learning paradigm (Bittermann *et al*. 1983; Menzel and Müller 1996). In a classical conditioning protocol bees learn to associate an odour (conditioned stimulus) with sucrose presentation (unconditioned stimulus; US). During conditioning an odour is presented shortly before stimulating the antenna with sucrose, which elicits the PER. The animal is then rewarded by applying sucrose to the proboscis, leading to an association of the odour with the reward. After a single learning trial up to 80% of the bees respond with a conditioned PER. An identified neurone (VUM_{mx1}) can mediate the US during olfactory conditioning in the honeybee (Hammer 1993). Immunohistological studies suggest that the VUM_{mx1} neurone belongs to a group of octopaminergic cells. It has been shown that electrical stimulation of the VUM_{mx1} neurone (Hammer 1993) or the injection of its putative transmitter octopamine, either into the antennal lobes or the calyces of the mushroom bodies (Hammer and Menzel 1998), can substitute for the US during olfactory conditioning.

To understand the molecular mechanisms that are controlled by the octopaminergic system it is necessary to characterize the respective octopamine receptors in the bee. Here we describe the molecular cloning and functional characterization of the first octopamine receptor from honeybee brain. The encoded protein is 587 amino acid residues in length and shares ~60% amino acid similarity with octopamine receptors from *Drosophila* (Han *et al*. 1998) and *Lymnea* (Gerhardt *et al*. 1997a). The functional characterization of this octopamine receptor should help to identify its role in honeybee neuromodulation and learning.

Materials and methods

Isolation of cDNA clones and sequencing

A cDNA library from *Apis mellifera* brains was constructed in λ ZAP II (Stratagene, La Jolla, CA, USA) and screened with a 900-bp *Eco*RI/*Xba*I restriction fragment of the AmBAR1-cDNA clone (Ebert *et al*. 1998; Kokay *et al*. 1999) as a probe. Hybridization was performed under high stringency in 5 × SET (20 × SET contains 3 M NaCl, 0.4 M Tris-HCl, pH 7.5, 0.02 M EDTA), 5 × Denhardt's solution (100 × Denhardt's contains 2% bovine serum albumin, 2% Ficoll 400, 2% polyvinylpyrrolidone), 100 μg/mL autoclaved herring testis DNA, 0.1% sodium dodecyl sulfate (SDS) and ~1×10^6 cpm/mL labelled probe at 61°C overnight. Filters were rinsed in 1 × SET, 0.1% SDS for 5 min at 22°C and twice for 30 min at 61°C. Plasmid DNA of positive clones was isolated using the *in vivo* excision protocol (Stratagene). Subcloning of restriction fragments was done into pBluescript SK(-) vector (Stratagene) by standard cloning techniques (Sambrook *et al*. 1989). Sequencing of restriction fragments used the thermo sequenase fluorescent-labelled primer cycle sequencing kit (Amersham-Pharmacia, Piscataway, NJ, USA) and the LICOR electrophoresis system (MWG Biotech, Ebersberg, Germany). The nucleotide sequence of Am*oa*1 has been submitted to the EMBL database (accession number AJ 547798).

Multiple sequence alignment and phylogenetic analysis

Biogenic amine receptor sequences for phylogenetic analysis from both *Drosophila* and *A. mellifera* were deduced from cDNA sequences in GenBank release 133.0. The *Drosophila* genomic DNA sequence was also searched using known biogenic amine receptors as query sequences to identify additional receptor homologues. TBLASTn searches, not filtered for low complexity, employing the Blosum 45 scoring matrix with a gap penalty of 16 : 1 were used to ensure that all homologues were identified. The results of the homology searches were combined with the computer-generated hypothetical protein predictions in GenBank to enhance the accuracy of receptor sequence prediction. All single nucleotide discrepancies were resolved in favour of the genomic DNA sequence. Multiple sequence alignments were performed, and highly divergent sequences at the amino and carboxyl termini and between TM5 and TM6 were trimmed from the sequences. Genetic distance between sequences was then calculated with ClustalX (Thompson *et al*. 1997) using the Blosum scoring matrix option. Neighbour joining trees were constructed in ClustalX using 1000-fold bootstrap re-sampling and the resulting trees were displayed graphically by Treeview (Page 1996) using the divergent muscarinic receptors as an outgroup. For clarity, two branches of 15% and 18% bootstrap support were collapsed to the base of the tree. A second tree was created from all pharmacologically defined tyramine and octopamine receptors, and from two additional orthologous receptors deduced from cDNA sequences. These sequences were from a range of insect species, a nematode and several molluscs. Alignment and tree construction parameters were the same as above.

Construction of pcAm*oa*1 expression vector

A truncated version of the Am*oa*1 cDNA containing a unique *Hind*III restriction site and the Kozak consensus motif (Kozak 1984) immediately 5′ to the initiating ATG codon was constructed by PCR. The following oligonucleotides were used: 5′-GATAAGCTT-CCACCATGCGATCCGTATTC and 5′-ATGGATCCTCAAGGT-CAA. The PCR product was digested with *Hind*III and *Xba*I. The Am*oa*1 cDNA in pBluescript SK(-) was digested with *Xba*I and *Eco*RI. Restriction fragments were gel purified and ligated into *Hind*III and *Eco*RI-cut pcDNA1.amp-vector (Invitrogen, Carlsbad, CA, USA). To monitor transfection efficiency and receptor protein expression, a haemagglutinin epitope-Tag (HA-Tag) was engineered to the 3′ end of the cDNA clone by standard cloning techniques. The resulting recombinant was named pcAm*oa*1-HA.

In situ hybridization

Hybridization to cryosections of adult honeybee brain was performed with digoxigenin-labelled riboprobes. Antisense and sense probes were transcribed using T7 and T3 RNA polymerase respectively from a cDNA fragment (1974–2092bp) encoding part of the third intracellular loop of the AmOA1 receptor, which had been cloned into pBluescript. Pre-hybridization was performed in 50% formamide, 5 × SSC (20 × SSC contains 3 M NaCl, 0.3 M sodium citrate, pH 7.4), 100 μg/mL autoclaved herring testis DNA, 50 μg/mL heparin and 0.1% Tween 20 at 45°C for 30 min. Hybridization was done in pre-hybridization solution containing 0.5 μg/mL digoxigenin-labelled probe overnight in a humidified chamber at 45°C. Washing was carried out in 50% formamide and 2 × SSC at 37°C for 60 min followed by two washes for 90 min each. For detection of hybrids, the sections were incubated with anti-digoxigenin antibody conjugated with alkaline phosphatase and stained with Nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) solution following the DIG Nucleic Acid Detection Kit (Roche Applied Science, Penzberg, Germany).

Heterologous expression of pcAm*oa*1-HA

Exponentially growing human embryonic kidney (HEK) 293 cells ($\sim 2 \times 10^5$ cells per 5 cm dish) were transfected with 10 μg pcAm*oa*1-HA by a modified calcium phosphate method (Chen and Okayama 1987). For Ca^{2+} fluorimetric experiments cells were co-transfected with pcAm*oa*1-HA and a gene encoding enhanced cyan fluorescent protein (ECFP; BD Biosciences, Heidelberg, Germany). Twenty hours after transfection the precipitate was washed off with phosphate-buffered saline (PBS) followed by PBS/1.34 mM EDTA. Cells were either transferred on to poly-L-lysine coated coverslips (for Ca^{2+} fluorimetry) or were left in the Petri dishes (for membrane preparations) and fed with fresh medium. Functional coupling of expressed receptors to intracellular signalling pathways was tested 24 h later.

Functional characterization of AmOA1 receptors

The ability of AmOA1 to trigger changes in $[Ca^{2+}]_i$ was monitored with the Ca^{2+}-sensitive fluorescence dye Fluo-4 (Molecular Probes, Eugene, OR, USA). Experiments were done on co-transfected HEK 293 cells which facilitated identification of AmOA1-expressing cells because they showed ECFP fluorescence (λ_{exc} 435 nm; λ_{em} 520–560 nm). Cells were incubated at 37°C in extracellular solution (ES; 150 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, 30 mM glucose, pH 7.4) containing 2 μM Fluo-4AM (Molecular Probes) and 0.02% Pluronic® F-127 (Molecular Probes). After 45 min cells were washed with dye-free ES. For receptor activation cells were superfused with ES containing different concentrations of octopamine or tyramine. A single-cell photon-counting system (PhoCal, Life Science Resources, Cambridge, UK) was used to measure $[Ca^{2+}]_i$-dependent changes in Fluo-4 fluorescence. Excitation wavelength was 480 nm (xenon lamp, 100 W; Nikon, Dusseldorf, Germany). Fluorescence emission was detected at 520–560 nm. The sampling rate of the photon-counting system was adjusted to 100 ms.

Assays to determine the ability of AmOA1 to activate adenylyl cyclase were performed after transient expression of pcAm*oa*1-HA in HEK 293 cells. Incubations with different ligands were performed at 37°C for 30 min in the presence of the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX; final concentration 10 μM). Cells were lysed by adding ice-cold ethanol (2 mL/dish). After 2 h at 4°C the lysate was transferred into an eppendorf cup and lyophilized. The amount of cAMP produced was determined using the TRK 432 cAMP assay kit (Amersham). Mean values of cAMP per Petri dish were determined in duplicate on two independent transfections.

Results

Molecular and structural properties of the octopamine receptor from honeybee brain

A cDNA fragment encoding TM6–7 of a GPCR was used to isolate full-length cDNA clones from head and brain-specific cDNA libraries (Blenau *et al*. 1998; Ebert *et al*. 1998; Kokay *et al*. 1999). The longest recombinant (Am*oa*1) consists of 3023 bp. The major open reading frame contains an initiation codon (ATG) at position 1058–1060 and is terminated by a translational stop codon (TGA; nucleotides 2819–2821). Nonsense codons are found in all three reading frames preceding the ATG codon. Interestingly, there are eight upstream ATG sequences in the 5′ non-translated region of the transcript. The open reading frames headed by these ATG sequences range from 18 to 153 nucleotides. The 3′ non-coding region of Am*oa*1 consists of 202 nucleotides and is terminated by a poly(dA) tail of 40 residues. The deduced amino acid sequence of Am*oa*1 (AmOA1, Fig. 1) consists of 587 residues with a calculated molecular weight of 66.5 kDa.

The deduced amino acid sequence of Am*oa*1 shows characteristic features of the GPCR superfamily (Strader *et al*. 1995; Valdenaire and Vernier 1997). The hydropathy profile (data not shown) reveals seven hydrophobic domains, which most probably serve as membrane-spanning segments (TM1–7; Fig. 1). The presence of highly conserved residues that contribute to ligand binding in biogenic amine receptors supports the hypothesis that AmOA1 belongs to the subfamily of biogenic amine receptors (Strader *et al*. 1995). The conserved residues include an Asp residue in TM3 (D_{145}) and Ser residues in TM5 (S_{251}ALGS_{255}), which are also present in AmOA1 (Fig. 1). AmOA1 contains three consensus sites for N-linked glycosylation (N-X-S/T). One site is located in the N-terminus (N_{47}AT) and two sites are present in the second extracellular loop (N_{214}MT, N_{224}TT). Five consensus sites for phosphorylation by protein kinase C (PKC) (S/T-X-R/K) are present within the intracellular loops IL1 and IL3 and in the C-terminus (Fig. 1). In addition, four consensus sites for phosphorylation by protein kinase A (RRP**S**$_{405}$RR, RRN**S**$_{409}$CES, KRR**T**$_{534}$NTL and RRG**S**$_{541}$) are present. The C-terminus of AmOA1 comprises 72 amino acids and harbours three cysteine residues (C_{526}, C_{528}, C_{530}) which might be a target for post-translational palmitoylation (O'Dowd *et al*. 1989; Jin *et al*. 2000).

Fig. 1 Deduced amino acid sequence of AmOA1. Amino acids are numbered beginning with the initiating methionine. The position of the last residue in each line is given in the right margin. The seven putative TMs are overlined. Three potential N-glycosylation sites (▼) as well as three cysteine residues for potential palmitoylation in the C-terminus (◇) are indicated. Amino acid residues that are implicated in ligand binding are shown as white letters on a black background. Potential phosphorylation sites for PKC (■) and protein kinase A (□) are also indicated.

When the AmOA1 sequence was used to query the non-redundant translated GenBank databases (release 133.0), a high degree of amino acid similarity with other insect and molluscan octopamine receptors was revealed. The greatest similarity (identical and conservatively substituted amino acid residues) of 66% exists between AmOA1 and the splice variant 1B of the *Drosophila* octopamine receptor (DmOA1B, GenBank accession no. AJ007617). The amino acid similarity between AmOA1 and the *Drosophila* octopamine receptor OAMB (Han *et al*. 1998) is 59%. To further define biogenic amine receptor relationships, we compared all known honeybee sequences, including that of AmOA1, to all *Drosophila* biogenic amine receptor sequences, either deduced from cDNA or predicted from the *Drosophila* genome. Six honeybee and 11 *Drosophila* receptor sequences were deduced directly from cDNA sequences whereas an additional 10 were predicted from genomic DNA. Two of these predicted sequences were excluded from the analysis because they were either highly divergent, pseudogenes or contained errors of prediction that could not be resolved.

A multiple sequence alignment of these 25 biogenic amine receptors was performed and used to build a phylogenetic tree (Fig. 2a). This tree groups AmOA1 with the two *Drosophila* octopamine receptor splice variants, DmOA1B and OAMB (Han *et al*. 1998). These sequences are most closely related to a clade containing the DAMB (Han *et al*. 1996) and AmDOP2 (Humphries *et al*. 2003) sequences. Both the DAMB and Am*dop*2 genes encode dopamine receptors unique to protostomes. Notably, AmOA1 is quite distinct from tyramine receptors (i.e. DmTYR and AmTYR1; Fig. 2a).

Phylogenetic analysis of all pharmacologically characterized octopamine and tyramine receptors (Fig. 2b) showed that AmOA1 clusters with the *Lymnea* and *Drosophila* sequences, LsOA1 (Gerhardt *et al*. 1997a), OAMB (Han *et al*. 1998) and DmOA1B, which is in complete agreement with the sequence homology search results.

Functional expression of Am*oa*1 cDNA in HEK 293 cells

To investigate the functional properties of AmOA1, HEK 293 cells were transiently transfected with pcAm*oa*1-HA. Octopamine receptors are known to increase intracellular cAMP and/or $[Ca^{2+}]_i$ concentrations (reviewed in Roeder 1999), so we analysed the effects of AmOA1 activation on these intracellular second messenger systems. To monitor Ca^{2+} signals, transfected HEK 293 cells were loaded with the calcium-sensitive dye Fluo-4. Superfusing the cells with low concentrations of octopamine (1 nM) did not change $[Ca^{2+}]_i$ (Figs 3a and c). An octopamine concentration of 50 nM, however, reproducibly induced oscillations in $[Ca^{2+}]_i$ (Figs 3a and b). In most of the cells analysed, the responses lasted as long as the ligand was present (Fig. 3a). In some experiments, cells responded to ligand application with a delay (Fig. 3b; 50 nM octopamine) and stopped signalling even in the continuous presence of the ligand (Fig. 3b;

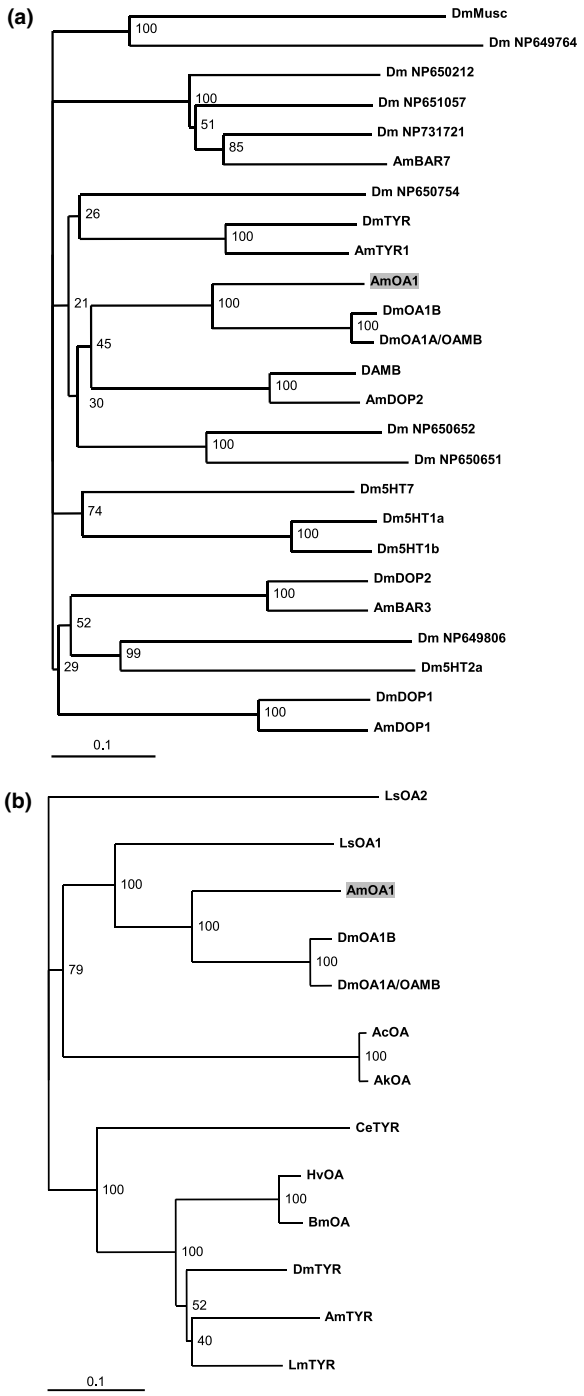

Fig. 2 Dendrogram of AmOA1 and protostomian biogenic amine receptors. (a) Phylogenetic comparison of all known *Apis* and *Drosophila* biogenic amine receptor sequences. The amino acid sequences were deduced from cDNA sequences except for eight of the *Drosophila* sequences, which were predicted from genomic DNA. Two additional likely biogenic amine receptor sequences were excluded from the analysis owing to sequence divergence and difficulty predicting a reliable protein sequence. AmOA1 was aligned with dopamine receptors from *Apis* (AmDOP1, accession no. (#)Y13427; AmDOP2, #AF498306; AmBAR3) and *Drosophila* (DAMB, #U61264; DmDOP1, #X77234; DmDOP2, #AAN15955); octopamine receptors from *Drosophila* (DmOA1A/OAMB, #AF065443; DmOA1B, #AJ007617); serotonin receptors from *Drosophila* (Dm5HT7, #P20905; Dm5HT1a, #P28285; Dm5HT1b, #P28286; Dm5HT2a, #CAA57429); tyramine receptors from *Apis* (AmTYR1, #AJ245824) and *Drosophila* (DmTYR, #M60789); a muscarinic receptor from *Drosophila* (DmMusc, #S05661) and hypothetical *Drosophila* proteins. (b) Phylogenetic relationships between octopamine and tyramine receptors of insects, molluscs and nematodes. AmOA1 was aligned with octopamine receptors from *Aplysia* (AcOA, #AAF37686; AkOA, #AAF28802), *Bombyx* (BmOA, #Q17232), *Drosophila* (DmOA1A/OAMB, #AF065443; DmOA1B, #AJ007617), *Heliothis* (HvOA, #Q25188) and *Lymnea* (LsOA1, #O77408; LsOA2, #O01670); tyramine receptors from *Apis* (AmTYR, #AJ245824), *Caenorhabditis* (CeTYR, #NM171978), *Drosophila* (DmTYR, #M60789) and *Locusta* (LmTYR, #Q25321). The numbers at the nodes of the branches represent the percentage bootstrap support for each branch. The scale bars allow conversion of branch lengths in the dendrogram to genetic distance between clades (0.1 = 10% genetic distance). Am, *Apis mellifera*; Ac/Ak, *Aplysia californica/kurodai*; Bm, *Bombyx mori*; Ce, *Caenorhabditis elegans*; Dm, *Drosophila melanogaster*; Hv, *Heliothis virescens*; Lm, *Locusta migratoria*; Ls, *Lymnea stagnalis*.

100 nM octopamine). The presence of the ligand, however, was necessary for the cellular responses. After stimulating the cells with 50 nM octopamine (Fig. 3b), the perfusion medium was changed for ligand-free ES, which led to the complete disappearance of Ca^{2+} signals (Fig. 3b; 'wash'). Adding 100 nM octopamine to the ES re-evoked the Ca^{2+} signals. The higher ligand concentration increased the frequency of the Ca^{2+} spikes (Fig. 3b) but did not change the signal amplitude. Similar observations were made with octopamine concentrations up to 300 nM (not shown). At high octopamine concentrations (1 μM) the cells responded with one large, slowly decaying Ca^{2+} signal (Fig. 3c). Once the cells had been treated with micromolar concentrations of octopamine they could not be stimulated with octopamine again (nanomolar to micromolar concentrations) even after long periods (≥ 15 min) of wash-out (not shown).

Application of tyramine in the concentration range used for octopamine activated Ca^{2+} signals only at high ligand concentrations (1 μM). Compared with the effect of octopamine at the same concentration, the tyramine effect was always delayed (Figs 3c and d). Octopamine (1 μM) and tyramine (1 μM) did not induce any Ca^{2+} response in non-transfected cells (data not shown).

To examine the ability of AmOA1 to activate adenylyl cyclase, transfected cells were incubated with increasing concentrations of octopamine and tyramine (1 nM to 10 μM) and with 1 μM dopamine and serotonin in the presence of the phosphodiesterase inhibitor IBMX (10 μM). Octopamine stimulated cAMP production only moderately at high concentrations (≥ 1 μM) (Fig. 4). Tyramine, serotonin and dopamine did not change cAMP levels in transfected cells. As was described earlier, non-transfected HEK 293 cells did

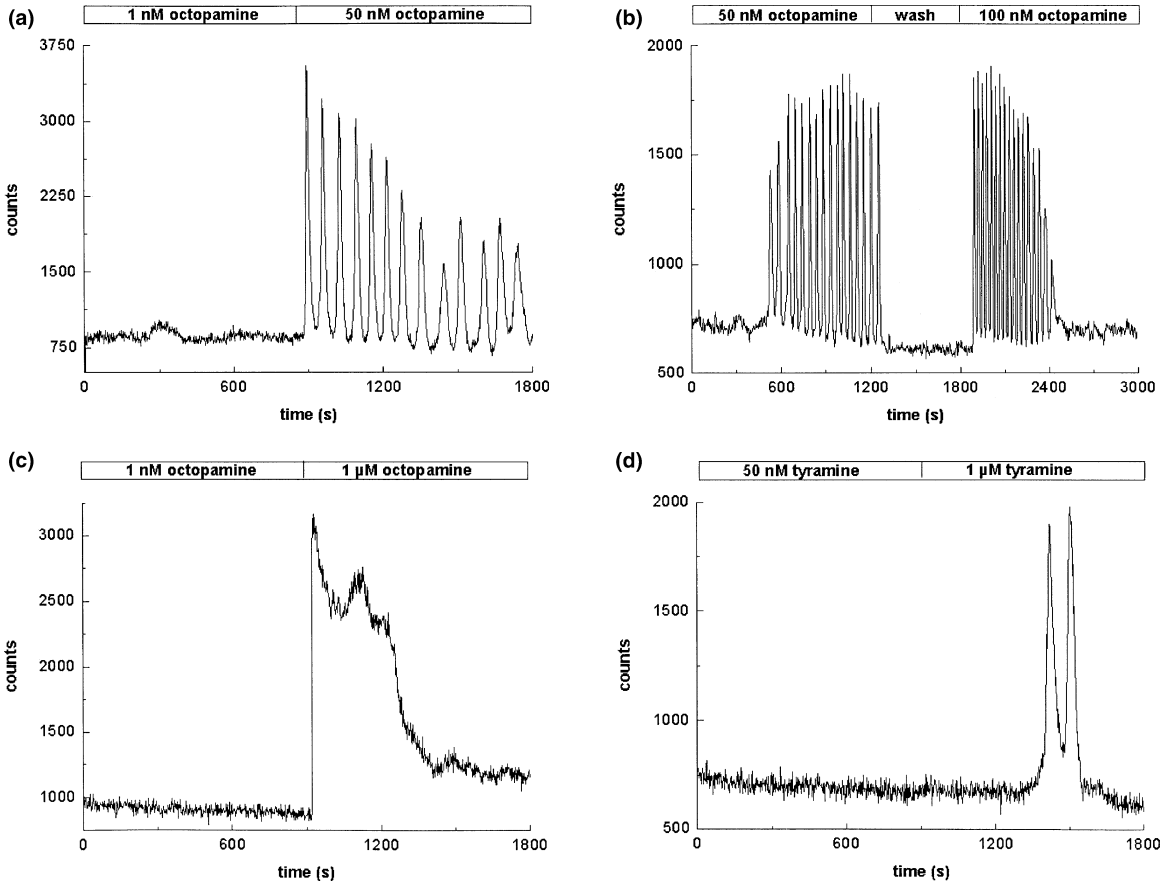

Fig. 3 Agonist modulation of $[Ca^{2+}]_i$ in transiently Am*oa*1-HA-transfected HEK 293 cells. Octopamine induced transient increases in $[Ca^{2+}]_i$. Calcium responses of individual cells expressing the AmOA1 receptor during stimulation with octopamine or tyramine are shown. Cells were loaded with the Ca^{2+}-sensitive dye Fluo-4. Relative fluorescence intensity is depicted as counts per 100 ms on the ordinate. Application of the ligands is indicated above the signals. In the following descriptions, the number of responding cells as a proportion of the total number of cells analysed is given in parentheses. (a) Superfusion with 1 nM and 50 nM octopamine (10 of 13). (b) Superfusion with 50 nM octopamine. The perfusion was changed for a ligand-free test solution and subsequently for a test solution containing 100 nM octopamine (five of five). (c) Superfusion with 1 nM and 1 µM octopamine. Concentrations ≥50 nM led to oscillations in $[Ca^{2+}]_i$ whereas 1 µM octopamine caused a large increase in $[Ca^{2+}]_i$ which decayed slowly (four of four). (d) Cells were superfused with 50 nM and 1 µM tyramine (five of seven).

not show a cAMP response to any of these amines (Gotzes *et al*. 1994). However, because HEK 293 cells express endogenous β-adrenergic receptors (Gerhardt *et al*. 1997b), incubation of non-transfected and Am*oa*1-transfected cells with noradrenaline (10 µM) caused a significantly higher level of cAMP production (~19 pmol/dish; not shown) than was observed with octopamine (~2.5 pmol/dish; Fig. 4). From these results we conclude that activation of heterologously expressed AmOA1 with octopamine at physiological concentrations specifically causes $[Ca^{2+}]_i$ oscillations. The increase in intracellular cAMP concentration observed at high octopamine concentrations was most probably a secondary effect, induced by massive Ca^{2+} release.

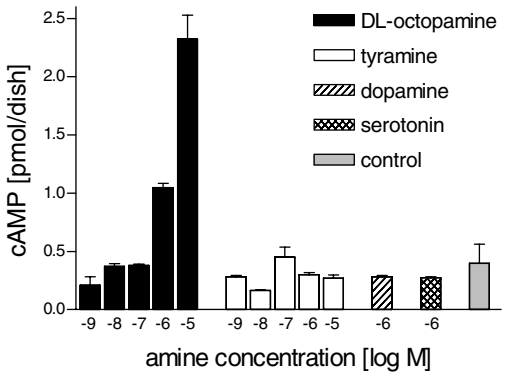

Fig. 4 Agonist modulation of intracellular cAMP in transiently Am*oa*1-HA-transfected HEK 293 cells. Agonist-induced increases in intracellular cAMP. Data are mean values of two independent experiments conducted in duplicate. Error bars indicate maximum and minimum values. Cells were incubated with IBMX only (10 µM) as a control.

Expression pattern of the Am*oa*1 gene

The distribution of Am*oa*1 mRNA was analysed by *in situ* hybridization to cryosections of adult worker bee brain. A

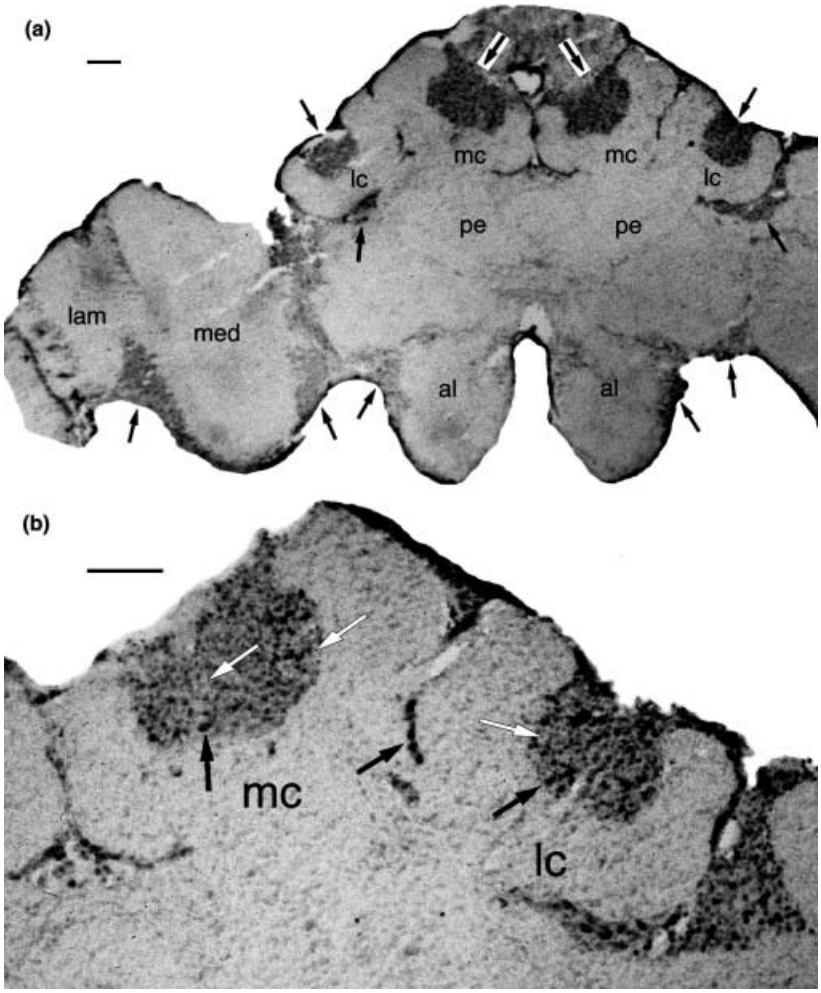

Fig. 5 *In situ* hybridization of Am*oa*1 antisense riboprobes to frontal sections of the honeybee brain. (a) Specific labelling is seen in the somata of many brain areas. The arrows indicate labelling of soma clusters in the brain. No specific labelling was obtained when a sense probe was used (data not shown).(b) Enlargement of the mushroom body calyces of one hemisphere. The non-uniformity of the signals in the somata of mushroom body intrinsic cells is clearly visible. Signals differ between very strong (black arrows) and weak (white arrows). Scale bars in both figures 100 μm. al, Antennal lobe; lam, lamina; lc, lateral calyx of the mushroom body; mc, median calyx of the mushroom body; med, medulla; pe, pedunculus of the mushroom body.

series of frontal sections was hybridized with digoxigenin-labelled riboprobes of either the antisense or the sense transcript. Labelling of cell somata was observed using the antisense probe only. Hybridization signals were present in many different soma clusters of the brain. Prominently labelled soma clusters in the brain include mushroom body intrinsic neurones (Fig. 5), cells belonging to the optic lobes and the deutocerebrum. This general result is similar to the mRNA distribution patterns previously described for dopamine (Blenau *et al*. 1998) and tyramine receptors (Blenau *et al*. 2000) from honeybee. Specific characteristics of Am*oa*1 labelling compared with Am*tyr*1 labelling are apparent in the soma cell cluster of mushroom body intrinsic cells. Am*oa*1 expression is not uniformly distributed among the somata of this cluster (Fig. 5b). Some somata exhibit very strong signals whereas others are only weakly stained. The distribution of differentially stained somata, however, does not correlate with expression of Am*oa*1 in distinct cell types of intrinsic mushroom body neurones. A similar observation was described for the *Drosophila* octopamine receptor OAMB (Han *et al*. 1998).

Discussion

In the present study we have identified and functionally characterized the first octopamine receptor from the honeybee, *A. mellifera*. Phylogenetic analysis of the deduced amino acid sequence shows that AmOA1 is a member of the subfamily of protostomian octopamine receptors. Activation of the heterologously expressed receptor by nanomolar concentrations of octopamine leads to oscillations in $[Ca^{2+}]_i$. The receptor encoding mRNA is abundantly expressed in many soma clusters of the honeybee brain, suggesting that AmOA1 plays a role in the processing of sensory information, antennal motor output and in higher-order brain functions.

Molecular properties of the AmOA1 receptor

Seven octopamine receptors have been functionally characterized so far, two from *Lymnea stagnalis* (Gerhardt *et al*. 1997a, 1997b), one from *Drosophila melanogaster* (Han *et al*. 1998), one from *Aplysia californica* (Chang *et al*. 2000), one from *Aplysia kurodai* (Chang *et al*. 2000), one

from *Heliothis virescens* and one from *Bombyx mori* (von Nickisch-Rosenegk *et al*. 1996). These receptors belong to three distinct clades, two of which contain octopamine receptors and one of which contains receptors that interact with tyramine and/or octopamine. Despite the fact that the AmOA1 receptor resides within a larger clade, which also contains the insect dopamine receptors DAMB and AmDOP2 (Han *et al*. 1996; Kokay *et al*. 1999; Humphries *et al*. 2003), we find no evidence that it is responsive to dopamine. The AmOA1 receptor shares the characteristic seven TM domain motif of GPCRs and possesses signature amino acid residues that are specifically implicated in ligand binding (Strader *et al*. 1995; Baldwin *et al*. 1997; Palczewski *et al*. 2000). The protonated amino group of octopamine probably pairs with the carboxyl group of Asp_{145} in TM3 of AmOA1. Serine residues (Ser_{251} and Ser_{255}) located in TM5 of AmOA1 are potential candidates for interaction with the hydroxyl group of the benzoyl ring of octopamine.

Signal transmission from activated GPCRs to intracellular effector systems is usually mediated by binding to specific heterotrimeric G proteins. The physical interaction of the receptor with its associated G protein is determined by amino acid residues located in the third intracellular loop and the C terminus of the receptor protein (Bourne 1997; Wess 1997). However, the amino acid sequence homology between the intracellular loops of AmOA1 and published octopamine receptor sequences is too low to allow deduction of signalling capabilities of the AmOA1 receptor by sequence comparison alone.

Functional coupling to intracellular second messenger pathways

To characterize the signalling properties of AmOA1, cDNA encoding the receptor was transiently transfected into HEK 293 cells. In contrast to various insect cell lines (Orr *et al*. 1992; Hu *et al*. 1994; Van Poyer *et al*. 2001; Näsman *et al*. 2002), these cells do not express endogenous octopamine receptors (Gotzes *et al*. 1994; Han *et al*. 1996, 1998; Blenau *et al*. 2000). Activation of heterologously expressed AmOA1 by octopamine led to Ca^{2+} oscillations (Fig. 3). The effect was specific for octopamine, because nanomolar concentrations of the ligand were sufficient to evoke the signal, whereas micromolar concentrations of tyramine were necessary to cause a delayed, short-lasting rise in $[Ca^{2+}]_i$. In addition to these Ca^{2+} signals, transfected cells also displayed a very small increase in intracellular cAMP concentration. The cAMP response, however, was only observed when high concentrations of octopamine were applied. These results suggest that the increase in $[Ca^{2+}]_i$ is the primary cellular response to AmOA1 activation. It has been shown that heterologously expressed GPCRs can activate different intracellular signalling systems, depending on the cell line used for expression and the agonist used for receptor stimulation (Robb *et al*. 1994; Reale *et al*. 1997; Sidhu and Niznik 2000). In the case of AmOA1, however, we hypothesize that the massive increase in $[Ca^{2+}]_i$ caused by stimulation with high concentrations of octopamine activates adenylyl cyclase in a secondary reaction. Nevertheless, the cellular cAMP response is minute. Therefore, AmOA1 should be considered a member of the native OCT1 receptor gene family, which is known to mediate increases in cellular Ca^{2+} concentration (Evans and Robb 1993; Roeder 1999).

A distinctive feature of the calcium responses observed in pcAm*oa*1-transfected HEK 293 cells is the occurrence of Ca^{2+} oscillations. Such oscillations have been observed in numerous cell types and represent universal intracellular signals (Thomas *et al*. 1996; Berridge *et al*. 2000). We observed the first Ca^{2+} oscillations when the transfected cells were stimulated with 50 nM octopamine. Raising the octopamine concentration resulted in an increase in the oscillation frequency (Fig. 3). Similar Ca^{2+} oscillations have been reported for a rat metabotropic glutamate receptor, mGluR5 (Kawabata *et al*. 1996). A homologous glutamate receptor (mGluR1) generated a single transient Ca^{2+} signal. The Ca^{2+} signal depended on the phosphorylation status of the receptor proteins. The mGluR5 receptor possesses a consensus site for phosphorylation by PKC in the C-terminus. This site is absent in the mGluR1 receptor. A phosphorylation site was introduced into the mGluR1 receptor by site-directed mutagenesis. Activation of the 'mutated' mGluR1 receptor led to Ca^{2+} oscillations that were very similar to those generated by mGluR5 (Kawabata *et al*. 1996). A potential PKC phosphorylation site is also present in the C-terminus of AmOA1. It will be interesting to test by site-directed mutagenesis whether phosphorylation of AmOA1 is necessary for shaping the Ca^{2+} signals.

Functional implications of the AmOA1 receptor

Octopamine is an important neuromodulator in insects. In the honeybee brain, five clusters of approximately 100 octopamine-immunoreactive somata have been identified (Kreissl *et al*. 1994). These neurones project into all regions of the brain and suboesophageal ganglion except for the mushroom body pedunculi and parts of the α- and β-lobes (Kreissl *et al*. 1994).

We investigated the expression pattern of the AmOA1 encoding mRNA in the bee brain. The gene is abundantly expressed in all major regions of the brain. This expression pattern differs substantially from the pattern reported for the *Drosophila* OAMB receptor gene (Han *et al*. 1998), although it is possible that the difference is quantitative rather than qualitative. OAMB-encoding mRNA is highly enriched in the somata of intrinsic mushroom body neurones of *Drosophila*. Furthermore, only scattered expression was detected in the ellipsoid body of the central complex and some somata of the medulla (Han *et al*. 1998).

In the honeybee and in *Drosophila,* the mushroom bodies are involved in olfactory learning and memory formation (Erber *et al*. 1980; De Belle and Heisenberg 1994; Menzel *et al*. 1994; Meller and Davis 1996). Specificity of gene expression in this neuropil might therefore indicate involvement of the receptor in learning processes (Han *et al*. 1998). In the bee, the specific role of octopamine during learning was demonstrated in the mushroom bodies by microinjection of octopamine, which can substitute for the US during olfactory PER conditioning (Menzel *et al*. 1994; Hammer and Menzel 1998). It is important to note that similar effects were found after octopamine injections into the antennal lobes, which also participate in olfactory memory formation (Erber *et al*. 1980; Hammer and Menzel 1998).

A number of experiments with microinjections have shown that octopamine can specifically modify behavioural and neuronal responses in different neuropils of the bee brain. Octopamine injections into the optic lobes can enhance visual antennal reflexes (Erber *et al*. 1993; Erber and Kloppenburg 1995) and change the properties of motion-sensitive interneurones (Kloppenburg and Erber 1995). Octopamine injections into the dorsal lobe can enhance the activity of antennal motoneurones whose somata and dendrites are located in this brain area (Pribbenow and Erber 1996).

The Am*oa*1 gene is expressed in cells of the mushroom bodies, the antennal lobes and the optic lobes. Therefore, the AmOA1 receptor is probably involved in the processing of olfactory and visual information. Activation of the heterologously expressed AmOA1 receptor leads to an increase in $[Ca^{2+}]_i$. Because a low ligand concentration is required to evoke Ca^{2+} signals, we hypothesize that the increase in $[Ca^{2+}]_i$ is the primary cellular response mediated by AmOA1. In summary, the distribution pattern of the Am*oa*1 transcript and the functional properties of the heterologously expressed AmOA1 receptor strongly suggest that this receptor is involved in processing sensory information and in mediating higher-order brain functions in the honeybee.

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Der Serotonin-5-HT$_7$-Rezeptor der Honigbiene

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Abstract

The biogenic amine serotonin (5-HT) plays a key role in the regulation and modulation of many physiological and behavioral processes in both vertebrates and invertebrates. These functions are mediated through the binding of the neurotransmitter to 5-HT receptors, of which 13 subtypes have been characterized in vertebrates. In the honeybee, which is an important model organism for studying the neural basis of behavior, 5-HT is linked to higher-order behavioral functions. We have isolated a cDNA from the honeybee *Apis mellifera* (Am*5-ht*7) sharing high similarity to the 5-HT_7 receptor family. Expression of the Am5-HT_7 receptor in HEK 293 cells results in an increase in basal cAMP levels over non-transfected HEK 293 cells suggesting that Am5-HT_7 is expressed as a constitutively active receptor. Serotonin application elevates cAMP levels in a dose-dependent manner (EC_{50} = 1.1-1.8 nM). The Am5-HT_7 receptor is also activated by the agonist 5-CT. Methiothepin acts as an inverse agonist. Receptor expression has been investigated by RT-PCR, *in situ* hybridization, and Western-blotting experiments. Receptor mRNA is expressed in perikarya of various brain neuropils, including mushroom body neurons, and in peripheral organs. Thus, Am5-HT_7 is probably involved in sensory processing and in higher-order brain functions and may sub-serve some of the actions of 5-HT on peripheral organs, such as muscles and Malpighian tubules. This study marks the first molecular characterization of a serotonin receptor in the model organism honeybee and should facilitate the further analysis of the role(s) of the receptor in mediating the various central and peripheral effects of 5-HT.

Introduction

The honeybee, *Apis mellifera,* is one of the most popular model organisms for studying insect learning (Stollhoff et al., 2005; for reviews, see: Hammer and Menzel, 1995; Menzel and Müller, 1996) and division of labor within insect societies (for a review, see: Page and Erber, 2002). An important class of substances that control and modulate these phenomena are the biogenic amines (for reviews, see: Bicker, 1999; Blenau and Baumann, 2001; Page and Erber, 2002). Biogenic amines mediate their cellular effects by binding to G protein-coupled receptors (GPCRs), which are integral membrane proteins having seven transmembrane (TM) domains (for reviews, see: Vernier et al., 1995; Blenau and Baumann, 2001). So far, three dopamine receptors (Blenau et al., 1998; Humphries et al., 2003; Mustard et al., 2003; Beggs et al., 2005), one tyramine receptor (Blenau et al., 2000, Mustard et al., 2005), and one octopamine receptor (Grohmann et al., 2003) of the honeybee have been cloned and functionally characterized. No molecular data on serotonin receptors of the honeybee is available to date, although serotonin (5-hydroxytryptamine, 5-HT) is present in large quantities in the CNS of the honeybee (Taylor et al., 1992; Wagener-Hulme et al., 1999; Schulz and Robinson, 1999). The distribution of serotonergic neurons has been intensively studied in the brain of worker honeybees (Schürmann and Klemm, 1984; for a review, see: Bicker, 1999). A small set of ~75 cell bodies gives rise to neuronal branches that arborize into most parts of the brain neuropil. The adult set of serotonergic neurons consists of both persistent embryonic neurons and neurons that differentiate during pupal metamorphosis. Behavioral experiments have shown that 5-HT reduces or blocks rhythmic behavior and reflexes in the bee (Erber et al., 1993; Erber and Kloppenburg, 1995; Kloppenburg and Erber, 1995; Pribbenow and Erber, 1996; Blenau and Erber, 1998). Serotonin also has profound

effects on honeybee learning and memory as it impairs the acquisition and retrieval of learned behavioral patterns (Bicker and Menzel, 1989; Menzel et al. 1999).

Serotonin-sensitive binding sites have been characterized in membrane preparations of honeybee brains (Blenau et al., 1995). However, the pharmacological properties of insect and mammalian receptors differ considerably (Blenau et al., 1995). Thus, it is almost impossible to classify insect receptors according to existing mammalian schemes on the basis of pharmacological profiles (Tierney, 2001). Initial autoradiographic studies with [^{3}H]5-HT have shown that 5-HT-receptor density is low in the antennal lobes but high in the calyces of the mushroom bodies (Erber et al., 1993). Obviously, this approach does not allow individual subtypes of 5-HT receptors to be distinguished.

In the present study, we have identified and functionally characterized an *Apis mellifera* 5-HT_7 receptor (Am5-HT_7) that is widely expressed in the CNS and in peripheral tissues. When stably expressed in HEK 293 cells, Am5-HT_7 is constitutively active resulting in an elevated basal cAMP level ($[cAMP]_i$). Serotonin and 5-HT receptor agonists cause dose-dependent increases in $[cAMP]_i$ in transfected cells. These data strongly suggest a role for Am5-HT_7 in sensory processing, higher-order brain functions, and physiological tuning.

Materials and Methods

Cloning of Am*5-ht*7 cDNA

Degenerate primers (sense: 5´-CGCGAATTCCGATCTGYTGGYTICCITTYTT-3'; antisense: 5´-GCGGAATTCGGATCGYRTADATIAYIGGRTT-3') corresponding to the highly conserved amino-acid sequences CWLPFF and NP(V/I)IY(T/A), respectively, of biogenic-amine receptors were designed to amplify receptor fragments. The polymerase chain reaction (PCR) was performed on a honeybee-brain cDNA library (Stratagene), custom-made from poly(A)+ RNA purified from the central brain, mushroom bodies, central body, and deutocerebrum of *Apis mellifera carnica* (kindly provided by Dr. D. Eisenhardt, Free University Berlin). Amplification was carried out for 2.5 min at 94°C (1 cycle), followed by 35 cycles of 40 s at 94° C, 1 min at 50° C, 1 min at 72° C, and a final extension of 10 min at 72° C. A fragment of ~160 bp was amplified and cloned into pBluescript SK(-) vector (Stratagene, Amsterdam, Netherlands). The fragment was used to screen ~1×10^6 λ-phages of the cDNA library at high stringency as described previously (Blenau et al., 1998; Grohmann et al., 2003). Plasmid DNA of positive clones was isolated following the *in vivo* excision protocol (Stratagene). Restriction fragments were sub-cloned into pBluescript SK(-) vector by standard cloning techniques (Sambrook et al., 2001). Sequencing was performed by AGOWA (Berlin, Germany) and showed that the cDNA was incomplete at the 5' end. Therefore, we re-screened the library with a ~200 bp *Eco* RI/*Xho* I restriction fragment from the 5' end of the longest recombinant. This led to the isolation of the full length clone (Am*5-ht*7). The nucleotide sequence of Am*5-ht*7 has been submitted to the EBI database (accession no. AM076717).

Multiple sequence alignment and phylogenetic analysis

Amino-acid sequences used for phylogenetic analysis were identified by protein-protein BLAST searches of the NCBI databases (BLASTP, NCBI database) with the deduced amino-acid sequence of Am*5-ht*7 (Am5-HT_7) as "bait". Multiple sequence alignments of the complete amino-acid sequences were performed with BioEdit (version 5.0.9; Hall, 1999). The genetic distance between sequences was calculated with ClustalX (version 1.81; Thompson et al., 1997) by using the Gonnet series protein weight matrix option. A neighbor-joining tree was constructed with ClustalX by using 1000-fold bootstrap re-sampling. The resulting tree was displayed graphically by TreeView (Page, 1996).

Reverse transcription-PCR amplification of Am5-*ht*7 fragments

Total RNA was isolated from retinas, optic lobes, central brains, flight muscle, and Malpighian tubules of worker honeybees with TRIZOL™ LS (Invitrogen, Karlsruhe, Germany). The samples were either digested with DNase I (Ambion, Huntingdon, UK) to degrade contaminating genomic DNA or with DNase I and RNase Cocktail (Ambion) for controls. Am*5-ht*7-specific fragments were amplified from 200 ng total RNA by using the SuperScript One-Step RT-PCR System (Invitrogen). The sense primer was 5'-GCGCGTAGGATAGTTCTGG-3' and the antisense primer was 5'-CAATCGTCGACTTGGTCGATG-3'. The primers are located in exons V and VI, which are separated by an intron of >4.7 kb. Amplification was as follows: cDNA synthesis and denaturation with 1 cycle of 50°C for 30 min, 94°C for 2 min; PCR amplifications with 35 cycles of 94°C for 40 s, 50°C for 40 s, 72°C for 40 s, and a final extension at 72 °C for 10 min. Amplified products were analyzed by agarose-gel electrophoresis.

In situ hybridization

Hybridization to cryosections of adult worker-honeybee brains was performed with digoxigenin-labeled riboprobes as described earlier (Blenau et al., 1998, 2000; Grohmann et al., 2003). Antisense and sense probes were transcribed by using T7 and T3 RNA polymerase (Roche, Mannheim, Germany), respectively, from a 337 bp cDNA fragment (bp 566-903) encoding part of the third intracellular loop of the Am5-HT_7 receptor. Hybridization was for 12 h at 42 °C in a solution containing 50% formamide, 5× SSC (saline-sodium citrate; 20× SSC = 3 M NaCl, 0.3 M sodium citrate, pH 7.4), 100 µg/ml salmon sperm DNA, 50 µg/ml heparin, 0.1% Tween 20, and 0.5 µg/ml digoxigenin-labeled probe. Washing was carried out for 1 h at 37°C in 50% formamide/2× SSC followed by two washes of 90 min each. For the detection of hybrids, sections were incubated with anti- digoxigenin antibody conjugated with alkaline phosphatase (1:500; Roche) and stained with NBT/BCIP solution following the instructions of the DIG Nucleic Acid Detection Kit (Roche).

Antibody production and purification

Antibodies were raised against a fusion protein containing the C-terminus of Am5-HT_7. The fragment was amplified by PCR by using 5'-TTTGAATTCCTGAACAGGGATTTTCG-3' and 5'-AAAAAGCTTCAGGAAGCTCTCATTAG-3' as sense and antisense primers, respectively. The fragment was cloned into pMAL-c2X vector (New England Biolabs, Frankfurt, Germany). The construct was called pMAL-CT. The fusion protein (MBP-CT) was over-expressed in *E. coli* BL21 CP and purified by amylose affinity-chromatography (New England Biolabs). The antiserum was raised by the Pineda-Antikörper-Service (Berlin, Germany).

For purification of polyclonal anti Am5-HT_7 antibodies, the C-terminus of Am5-HT_7 was cloned into pET-30a vector (Novagen, Darmstadt, Germany) and over-expressed (HIS-CT).

Approximately 1.6 mg purified HIS-CT was coupled to a HiTrap NHS-activated HP column (Amersham Biosciences, Freiburg, Germany). Antibodies from 50 ml antiserum were affinity-purified by standard protocols.

Western-blot analysis

Honeybee brains were homogenized in 5× sample buffer (Roth, Karlsruhe, Germany) and incubated for 5 min at 95°C. Proteins were separated by SDS-PAGE on 10% or 12% gels. Proteins were transferred to nitrocellulose (Schleicher & Schuell BioScience, Dassel, Germany) or polyvinylidene fluoride membranes (Roth). Membranes were blocked with 5% dry milk in Tris-buffered saline containing Tween 20 (TBS-T; 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) for 30 min at room temperature. Membranes were probed with affinity-purified anti Am5-HT_7 antibodies (dilution 1:5,000 in TBS-T) for 3 h at room temperature. For controls, Am5-HT_7 antibodies were pre-absorbed to MBP-CT (5–15 µg) before immunostaining. Membranes were washed with TBS-T, followed by incubation with a secondary antibody conjugated to horseradish peroxidase (1:10,000; American Qualex, La Mirada, USA). Signals were visualized with an enhanced chemiluminescence detection system (ECL; Super Signal West Pico Chemiluminescent Substrate; Pierce, Rockford, USA).

Western blots with membrane fractions of human embryonic kidney cells (HEK 293) expressing Am5-HT_7-HA receptors (see below) were incubated with specific anti HA-antibodies (1:3000; Roche) in phosphate-buffered saline containing 0.1% Tween 20 (PBS-T). Membranes were washed with PBS-T, followed by incubation with secondary antibodies (1:5000; anti-rat-HRP; Dianova, Hamburg, Germany) for 1 h. Signals were visualized by ECL.

Construction of pcAm5-*ht*7-HA expression vector

A truncated version of the Am5-*ht*7 cDNA containing the Kozak consensus sequence (Kozak, 1984) immediately 5' to the initiating ATG codon was constructed by PCR. To monitor transfection efficiency and receptor protein expression, a hemagglutinin epitope-Tag (HA-Tag) was engineered to the 3' end of the cDNA. PCR was performed with a sense primer 5´-TTTAAGCTTCCACCATGGAGGGGAAGGACGC-3' and the antisense primers, 5´-TTTTCTAGATTAAGCGTAGTCTGGGACGTCGTATGGGTACAGGAA 3' (first-round PCR) and 5´-TCGTATGGGTACAGGAAGCTCTCATTAGG-3' (second-round PCR). The PCR product was digested with *Hind* III and *Xba* I and subcloned into pcDNA3.1(+) vector (Invitrogen) yielding pcAm*5-ht*7-HA.

Functional expression of Am5-HT_7-HA and preparation of membrane proteins from transfected cells

Approximately 8 µg pcAm*5-ht*7-HA vector was introduced into exponentially growing (~2×10^5 cells per 5 cm Petri dish) HEK 293 cells by a modified calcium phosphate method (Chen and Okayama, 1987). Stably transfected cells were selected in the presence of the antibiotic G418 at 0.8 mg/ml. Isolated foci were propagated and analyzed for expression of Am5 HT_7-HA by Western blot (see above). Briefly, cells were lysed in 10 mM NaCl, 25 mM HEPES, pH 7.5, 2 mM EDTA, 1 µg/ml Pefabloc. Membranes were collected by centrifugation and re-suspended in 200 mM NaCl, 50 mM HEPES, pH 7.5, 1 µg/ml Pefabloc. Solubilization of membrane proteins was achieved by adding the detergent 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) (1% final concentration). For deglycosylation, samples were incubated with PNGase F (New England Biolabs).

Functional characterization of Am5-HT_7 receptors

Assays to determine the ability of Am5-HT_7-HA receptors to stimulate adenylyl-cyclase activity were performed as described earlier (Grohmann et al., 2003; Balfanz et al., 2005). Am5 HT_7-expressing cells were grown in minimal essential medium (MEM + GlutaMAX^{TM}I, Invitrogen) with 2% (v/v) Ultroser G (Ciphergen, Cergy-Saint-Christophe, France) and antibiotics. Incubations were performed at 37°C for 30 min in the presence of the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX; final concentration 10 µM). Cells were lysed by adding 1 ml ice-cold ethanol. After 2 h at 4 °C, the lysate was transferred to an Eppendorf cup and lyophilized. The amount of cAMP produced was determined by using the TRK 432 cyclic AMP assay kit (Amersham Biosciences). Mean values of cAMP/mg protein were determined from two independent measurements performed in duplicate. Data were processed by using PRISM 4.01 software (GraphPad, San Diego, USA).

Results

Structural features of the Am5-HT$_7$ receptor and the Am*5-ht*7 gene

A cDNA fragment encoding transmembrane segments (TM) 6 – 7 of a GPCR was amplified from honeybee-brain cDNA and used to isolate a full-length cDNA clone. The longest recombinant (Am*5-ht*7) consists of 4,097 bp. The open reading frame of Am5-*ht*7 encodes a protein of 501 amino acids (Am5-HT$_7$), with a calculated molecular weight of 56.1 kDa. The translation initiation codon (ATG; position 1,195-1,197) is preceded by nonsense codons in all three reading frames. The longest open reading-frame is terminated by a translational stop codon (TGA; position 2,698-2,700). The 3' non-coding region consists of 1,398 nucleotides and is terminated by a poly(dA) tail of 16 residues.

The deduced amino-acid sequence of Am*5-ht*7 (Fig. 1) displays characteristic properties of GPCRs (Strader et al., 1995). The hydropathy profile (not shown) contains seven stretches of sufficient length to serve as membrane-spanning segments (TM1-7). Consensus sites for N-linked glycosylation are located in the N-terminus (N_{16}LT) and in the first extracellular loop (N_{106}WS; see Fig. 1). Three consensus sites for phosphorylation by protein kinase C (S/TXR/K) are located at positions T_{154}, T_{296}, and S_{335} (Fig. 1).

In addition, Am5-HT$_7$ contains amino-acid residues that are highly conserved between members of the biogenic-amine receptor family. The aspartic acid residue in TM3 (D_{121}) might bind to the protonated amino group of the ligand. Ligand-binding is further improved by hydrogen bonds formed between the hydroxyl group of a serine residue in TM5 (S_{210}) and the hydroxyl group of serotonin. Interestingly, the tyrosine residue of the highly conserved "DRY" motif at the C-terminus of TM3 of GPCRs is replaced by phenylalanine (F_{140}). Within TM6, the consensus sequence F_{381}XXXWXP is conserved among many non-peptide receptors. For most amine receptors, this motif is followed by a pair of phenylalanine

residues. The second phenylalanine is unique to aminergic receptors and is conserved in Am5 HT_7 (F_{389}). As is known from other GPCRs (Barak et al., 1994), the N_{423}PXXY motif in TM7 may participate in agonist-mediated sequestration and resensitization of the Am5-HT_7 receptor. The C-terminus of Am5-HT_7 comprises 71 residues. It contains two cystein residues ($C_{446,}$ C_{469}) that are potential targets for post-translational palmitoylation (Jin et al., 2000). In addition, Am5-HT_7 contains a PDZ-domain binding motif (E_{498}SFL) at its C-terminus.

Comparison of the deduced amino-acid sequence of Am5-*ht*7 with NCBI data bases identified several insect and mammalian serotonin-receptor orthologs. Highest sequence similarity exists to the 5-HT_7 receptor from *Aedis aegypti* (Aae5-HT_7; Pietrantonio et al., 2001) and the 5-HT_7 receptor from *Drosophila melanogaster* (Dm5-HT_7; Witz et al., 1990). The overall amino-acid similarity (identical and conservatively substituted amino acids) between Am5-HT_7 and Aae5-HT_7 and Dm5-HT_7 receptors is 56% and 54%, respectively.

A multiple sequence alignment of Am5-HT_7 and insect 5-HT receptors and of two phenolamine receptors was used to calculate a phylogenetic tree (Fig. 2). The Am5-HT_7 receptor clusters with Dm5-HT_7, Aae5-HT_7, and an uncharacterized receptor from *Anopheles gambiae* in a distinct clade. The next closely related clade contains, among others, the receptors Dm5-HT_{1A} and Dm5-HT_{1B} from *Drosophila* (Saudou et al., 1992).

Analysis of the genomic organization of the Am5-*ht*7 gene showed that it consists of at least seven exons. These exons are separated by large introns (Fig. 3). The coding region of the receptor protein is spread over four exons. Splice donor and acceptor sites at the exon/intron borders follow the consensus sequence motifs of metazoan genes.

Expression pattern of the Am5-*ht*7 gene

The tissue-specific expression pattern of Am*5-ht*7 was determined by reverse transcription-PCR (RT-PCR; Fig. 4). Receptor fragments were amplified on RNA samples isolated from

central brains, flight muscle, and Malpighian tubules. Smaller amounts of PCR products were detected in samples prepared from the optic lobes and retinas. The fragments are unlikely to have arisen from genomic DNA, because the primers are located in two exons (VI and VII) separated by a large intron (>4.7 kb). In addition, controls treated with an RNase cocktail do not result in the amplification of any PCR products (Fig. 4, right panel).

The distribution of Am*5-ht*7 mRNA was also examined by *in situ* hybridization to cryosections of adult worker-honeybee brains (Fig. 5). Hybridization signals were observed in many neurons, including neurons of the optic lobes, mushroom body intrinsic neurons (Kenyon cells), and neurons of the deutocerebrum. This result is similar to the mRNA distribution patterns previously described for a dopamine (Blenau et al., 1998; Kurshan et al., 2003; Beggs et al., 2005), a tyramine (Blenau et al., 2000, Mustard et al., 2005), and an octopamine receptor (Grohmann et al., 2003) expressed in honeybee brain. No signals were observed with the sense probe (data not shown).

Generation and purification of an anti Am5-HT_7 antibody

We generated a polyclonal antiserum against the C-terminus of Am5-HT_7 as detailed in Materials and Methods. A Western blot of *A. mellifera* brain proteins (Fig. 6A) shows that the antibody recognizes a single band of ~66 kDa. The signal is lost when the antibody is pre-absorbed with the fusion protein used to generate the antibody. This result indicates that the antibody specifically recognizes the Am5-HT_7 receptor protein. The higher molecular weight (~66 kDa) compared with the value deduced from the Am5-*ht*7 cDNA (56.1 kDa) is probably attributable to glycosylation (see below) or other posttranslational modifications of the native receptor protein. A protein band of similar size (~66 kDa) was also detected on Western blots of proteins from Kenyon cells that had been isolated from adult worker honeybees and kept in

culture for ~24 h (kindly provided by Drs. B. Grünewald and D. Eisenhardt, Free University Berlin; data not shown).

Using the anti Am5-HT_7 antibody, we also examined the expression of the Am5-HT_7-HA protein in stably transfected HEK 293 cells (Fig.6B). On Western blots of membrane proteins from these cells, the antibody recognizes a single protein band of ~70 kDa. Deglycosylation of membrane proteins by PNGase F digest results in the occurrence of a protein band of ~56 kDa. Therefore, both native and heterologously expressed Am5-HT_7 receptors seem to be highly glycosylated. For the heterologously expressed receptor, similar results have been obtained with an anti HA antibody that detects the HA-Tag attached to the 3' end of Am5-HT_7 (Fig. 6C).

Functional expression of Am5-HT_7 in HEK 293 cells

For functional characterization of the Am5-HT_7 receptor, we generated cell lines that express the Am5-HT_7-HA receptor (cell line 1 and line 4). Several aspects of the data suggest that Am5 HT_7, like the human and *C. elegans* orthologs (Krobert and Levy, 2002; Hobson et al., 2003), is constitutively active. In comparison to non-transfected HEK 293 cells, the Am5-HT_7 cell lines possess an elevated basal (non-agonist stimulated) $[cAMP]_i$ (Fig. 7A, B and C).

To test for the ligand specificity of the Am5-HT_7 receptor, we analyzed the effects of a range of biogenic amines on cAMP production in non-transfected and Am5-HT_7-expressing cell lines (Fig. 7A). Only 5-HT could elicit an increase in $[cAMP]_i$ in transfected cells, but it was ineffective with respect to non-transfected cells. Dopamine, tyramine, and octopamine did not change $[cAMP]_i$ in cells expressing Am5-HT_7. Interestingly, the serotonin analog 5-CT, which is a selective agonist for mammalian 5-HT_1 and 5-HT_7 receptors, also caused an increase in $[cAMP]_i$ in cells expressing Am5-HT_7.

The dose-dependent effect of 5-HT on $[cAMP]_i$ in Am5-HT_7-expressing cell lines was examined with 5-HT concentrations ranging from 10^{-10} to 10^{-5} M (Fig. 7B). Half-maximal stimulation of cAMP production (EC_{50}) was observed with 1.06 nM 5-HT ($logEC_{50}$ = -8.97 ± 0.13, mean ± SE) and 1.75 nM 5-HT ($logEC_{50}$ = -8.76 ± 0.07, mean ± SE) for cell line 1 and cell line 4, respectively. The production of cAMP saturated at 5-HT concentrations of ≥50 nM (Fig. 7B).

Furthermore, the effects of two putative antagonists on 5-HT-stimulated cAMP production were assayed (Fig. 7C). 5-HT (10 µM)-mediated cAMP production was suppressed by methiothepin (10 µM), an antagonist at mammalian 5-HT_1, 5-HT_6 and 5-HT_7 receptors. Methiothepin also reduced the agonist-independent activity of Am5-HT_7 as displayed by $[cAMP]_i$ levels below base-line levels in both cell lines. This result suggests that methiothepin acts as an inverse agonist at the Am5-HT_7 receptor (Sprouse et al., 2004). In contrast, clozapine (10 µM), an antagonist at mammalian 5-HT_{1A}, 5-HT_{2C}, 5-HT_3, 5-HT_6, and 5-HT_7 receptors, did not block Am5-HT_7-mediated cAMP production. Taken together, the pharmacological data support the notion that Am5-HT_7 is a member of the serotonin 5-HT_7 receptor class and is well suited to modulate intracellular cAMP levels *in vivo*.

Discussion

In most animal phyla, serotonin modulates important patterns of behavior, including feeding, sexual, and aggressive behavior, and learning and memory (for reviews, see: Weiger, 1997; Meneses, 1999). In humans, 5-HT has been implicated in the etiology of numerous disease states, including depression, anxiety social phobia, schizophrenia, and obsessive-compulsive and panic disorders. In addition, 5-HT contributes to migraine, hypertension, pulmonary hypertension, eating disorders, and vomiting. In mammals, the diverse effects of 5-HT are mediated by at least 13 distinct GPCRs and one ligand-gated ion channel(s) (for reviews, see: Hoyer et al. 2002; Kroeze et al. 2002). The receptors are divided into seven distinct classes largely based on their structural and operational properties. The 5-HT_1 receptors couple preferentially to $G_{i/o}$ and inhibit cAMP formation; 5-HT_2 receptors couple preferentially to $G_{q/11}$ and cause the hydrolysis of inositol phosphates and the subsequent elevation of cytosolic $[Ca^{2+}]_i$; 5-HT_3 receptors are ligand-gated ion channels; 5-HT_4, 5-HT_6, and 5-HT_7 receptors all couple preferentially to G_s and promote cAMP formation. It is assumed that, in insects, the serotonergic system may be similarly complex (for a review, see: Tierney, 2001).

In the present study, a 5-HT_7 receptor cDNA has been successfully cloned from honeybee brain. Orthologous receptors have been previously cloned from other invertebrate species. The first 5-HT_7 receptor was cloned from *Drosophila* (Witz et al., 1990; Saudou et al., 1992). Later, 5-HT_7 receptors were cloned from mosquito (Pietrantonio et al., 2001; Lee and Pietrantonio, 2003) and *C. elegans* (Hobson et al., 2003). All these invertebrate receptors share pronounced sequence similarity to mammalian 5-HT_7 receptors.

All insect 5-HT_7 receptors contain an identical C-terminal sequence (ESFL). This motif nicely fits the consensus sequence of a class I PDZ-binding domain (XS/TXV/I/L; for a review, see: Hung and Sheng, 2002) and suggests that the receptors may be assembled into signaling complexes by scaffolding proteins. A similar motif (ASDV) has been identified in nematode

5-HT_7 receptors (Hobson et al., 2003). Human 5-HT_{2A} and 5-HT_{2C} receptors, which also contain a class I PDZ-binding domain at their C-termini, have recently been shown to interact with specific sets of PDZ proteins (Bècamel et al., 2004). At present, however, none of the invertebrate receptors has been tested for such interaction partners.

Am5-HT_7 was stably expressed in HEK 293 cells, which have been used successfully in previous studies to examine the pharmacological properties of cloned GPCRs of the honeybee (Blenau et al., 1998, 2000; Grohmann et al., 2003, Beggs et al., 2005). The Western-blotting analyses presented here have demonstrated that Am5-HT_7 is expressed in a glycosylated form in HEK 293 cells. Various biogenic amines have subsequently been tested for their ability to induce cAMP and/or Ca^{2+} responses in Am5-HT_7-expressing cell lines. Am5-HT_7 exclusively couples to adenylyl cyclase. 5-HT and 5-CT, potent agonists at human 5-HT_7 receptors (Bard et al., 1993; Thomas et al., 1998), both cause an increase in $[cAMP]_i$ in Am5-HT_7-expressing cell lines. Similar to many other vertebrate (for a recent reviews, see: Milligan, 2003) and invertebrate (Mustard et al., 2003; Clark et al., 2004) biogenic-amine receptors, 5-HT_7 receptors from man (Thomas et al., 1998; Krobert and Levi, 2002), *C. elegans* (Hobson et al., 2003), and *Apis mellifera* (this study) possess agonist-independent (constitutive) activity. Initially attributed to genetically engineered GPCRs expressed in heterologous environments, there is now increasing evidence that constitutive receptor activity is a biologically relevant phenomenon (Morisset et al., 2000). Although the exact function remains to be unraveled, it is assumed that constitutive receptor activation is suitable for controlling the magnitude of agonist-induced responses *in vivo*. In addition, constitutively activated GPCRs may be targeted by naturally occurring inverse agonists, which lower the basal activity of the receptor and thereby provide an additional mechanism of fine-tuning agonist responses (for a review, see: Milligan, 2003).

Amino-acid residues in close vicinity to the plasma membrane of the second and third intracellular loops and of the cytoplasmic tail of GPCRs have been shown to determine the efficacy of G protein activation (e.g., Alewijnse et al., 2000). In particular, the highly conserved "DRY" motif, located at the intracellular border of TM3 and the second intracellular loop, seems to play a pivotal role in signal transduction by GPCRs. Shapiro et al. (2002) have provided evidence for a strong ionic interaction between the arginine residue of the "DRY" motif and a glutamic-acid residue close to the intracellular end of TM6 in the rat 5-HT_{2A} receptor. The interaction stabilizes the inactive state of the receptor, and disruption of this interaction by mutagenesis produces a constitutively active receptor with increased potency for agonists (Shapiro et al. 2002; for a review, see: Kroeze et al., 2002). In the Am5-HT_7 receptor, the "DRY" motif is changed to "DRF", i.e. a polar residue (Y) is replaced by a hydrophobic amino acid. It is tempting to speculate that this transition contributes to the agonist-independent activity, as has recently been shown for a crustacean 5-HT_2 receptor by site-directed mutagenesis (Clark et al., 2004).

Clues to the possible functions of the Am5-HT_7 receptor might be obtained from its cellular localization. *In situ* hybridization has shown that the gene is expressed in all major regions of the adult honeybee brain. Similar expression patterns have been described for the AmDOP1 (Blenau et al., 1998; Kurshan et al., 2003), the AmDOP3 (Beggs et al., 2005), the AmTYR1 (Blenau et al., 2000; Mustard et al., 2005), and the AmOA1 (Grohmann et al., 2003) receptor. The expression of Am*5-ht7* in the optic lobes, the antennal lobes, and the mushroom bodies, which are generally believed to play an important role in insect learning (Hammer and Menzel 1995; Schwaerzel et al., 2003; Heisenberg 2003), suggests that the receptor contributes to neural pathways that are involved in the processing of a variety of sensory information and possibly higher-order brain functions. Results from RT-PCR have largely confirmed the expression pattern observed by *in situ* hybridization experiments. Furthermore, we have detected large amounts of PCR products in samples from Malpighian tubules. The

Aae5-HT$_7$ receptor of the mosquito, *Aedis aegypti,* is localized in tracheolar cells associated with Malpighian tubules (Pietrantonio et al., 2001), suggesting a role for serotonin in respiration and the coordination of the tubule-hindgut response during diuresis. In mammals, the highest amount of 5-HT$_7$ receptors is found in the brain (thalamus, hypothalamus, hippocampus, and frontal cortex), although 5-HT$_7$ receptors have also been detected in the periphery (e.g., spleen, kidney, heart, coronary artery, gastrointestinal tract; for a review, see: Hedlund and Sutcliffe, 2004).

The mammalian 5-HT$_7$ receptor has been linked to several physiological and pathophysiological phenomena. It is a mediator of 5-HT-induced hypothermia (Hedlund et al., 2003) and the 5-HT-induced phase resetting of the circadian clock within the suprachiasmatic nucleus of the hypothalamus (Lovenberg et al., 1993; Ehlen et al., 2001; Sprouse et al., 2004). The 5-HT$_7$ receptor has also been implicated in endocrine regulation and neuropsychiatric disorders (for a review, see: Hedlund and Sutcliffe, 2004). The agonist 8-OH-DPAT enhances learning consolidation, and evidence suggests that both 5-HT$_{1A}$ and 5-HT$_7$ receptors might be involved in this process (Meneses and Terron, 2001). Most interestingly, mice lacking 5-HT$_7$ receptors are specifically impaired in contextual learning and display decreased long-term synaptic plasticity within the CA1 region of the hippocampus (Roberts et al., 2004). Molecular knock-down technologies, such as the antisense technique (Fiala et al., 1999) and the RNA interference (RNAi) technique (Farooqui et al., 2003), are feasible in the honeybee brain. The application of such techniques in combination with behavioral experiments should certainly help in the elucidation of the physiological functions of the Am5-HT$_7$ receptor in the bee. An examination of whether the Am5-HT$_7$ receptor is involved in honeybee learning will be especially interesting.

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Figure Legends

Figure 1: Amino-acid sequence alignment of Am5-HT_7 and orthologous receptors from *Drosophila melanogaster* (Dm5-HT_7; accession no. (#)NP_524599) and *Aedis aegypti* (Aae5-HT_7; #AAG49292). Identical residues between all three sequences are shown as white letters against black, conservatively substituted residues are shaded. Dashes indicate gaps that were introduced to maximize homologies. Putative transmembrane domains (TM1-TM7) are indicated by gray bars. Potential N-glycosylation sites (●) and potential phosphorylation sites for PKC (*) are labeled. The amino-acid position is indicated at the right.

Figure 2: Phylogram of Am5-HT_7, insect serotonin, and phenolamine receptors. Alignment was performed with the complete amino-acid sequence of each receptor. The genetic distance was calculated with ClustalX (version 1.81). The receptor sequences, followed by their GenBank accession numbers (#), are listed below in the order illustrated: *Drosophila melanogaster* Dm5-HT_7 (#NP_524599), *Apis mellifera* Am5-HT_7 (#AM076717, this study), *Aedis aegypti* Aae5-HT_7 (#AAG49292), *Anopheles gambiae* ENSANGP00000012908 (Aga12908; #EAA08564), Dm5-HT_{1B} (#NP_523789), Dm5-HT_{1A} (#NP_476802), *Heliothis virescens* Hel5-HT (HVSEROK15; #CAA64863), *Papilio xuthus* Pxu5-HT (#BAD72868), *Bombyx mori* Bom5-HT (BMSERB150; #CAA64862), *A. gambiae* ENSANGP00000004842 (Aga4842; #EAA13071), *A. mellifera* octopamine 1 (AmOA1; #NP_001011565), *A. mellifera* tyramine 1 (AmTYR1; #CAB76374), Dm5-HT_2 (#NP_524223), *D. melanogaster* CG8007-PB (#AAN13390), and *A. gambiae* ENSANGP00000006270 (Aga6270; #EAA03663).

Figure 3: Genomic organization of the Am*5-ht7* gene. The honeybee genome version 2.0 assembly (http://www.hgsc.bcm.tmc.edu/projects/honeybee/) was screened with the Am*5-ht7*

cDNA sequence. The seven exons of the Am*5-ht*7 gene are distributed over six non-overlapping contigs: contig7294 (#AADG04007294), contig10277 (#AADG04010277), contig12189 (#AADG04012189), contig10013 (#AADG04010013), contig9553 (#AADG04009553), and contig13979 (#AADG04013979). Distances are given in kilobases (kb), and the distribution of the exons (I-VII) is indicated by horizontal lines. The (minimal) size of each intron is shown. Breaks indicate that the exact intron length is not yet known. Transcription is from left to right. The cDNA is displayed schematically. Numbers below the boxes indicate exon sizes in base pairs. Non-coding regions are displayed as white boxes. The START and STOP codons define the open reading frame (gray boxes) of the gene. The position of the seven transmembrane domains is highlighted by black boxes.

Figure 4: Expression of the Am*5-ht*7 gene in various tissues of *Apis mellifera*. The 100-bp DNA ladder is shown in lanes 1 and 8. PCR amplification performed on a pBluescript clone harboring the Am*5-ht*7 cDNA served as a positive control (lane 2). Consecutive lanes contain RT-PCR products amplified from RNA of the organs given above each lane. Amplified fragments have the expected size of 482 bp. Because of the inclusion of a large intron (>4.7 kb between exons V and VI, see Fig. 3), genomic DNA was not amplified. Amplification failed if samples were pre-digested with an RNase cocktail prior to RT-PCR (negative controls, lanes 9 to 13).

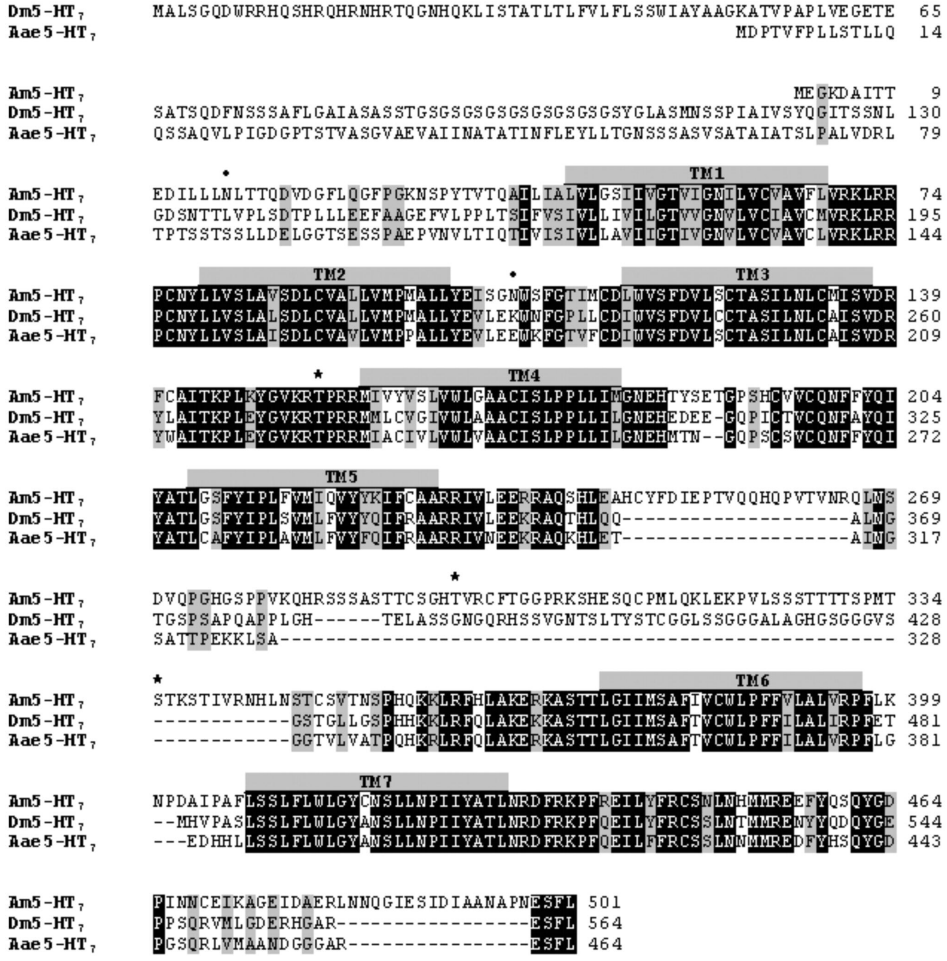
Figure 5: *In situ* hybridization of Am*5-ht*7 antisense riboprobes to frontal sections of the worker-honeybee brain. Specific labeling is seen in the somata of various brain areas, e.g., in the somata of mushroom body intrinsic neurons (black arrows), in somata surrounding the dorsal lobe and the sub-esophageal ganglion (gray arrows), and in somata of the optic lobes (white arrows). No specific labeling was seen when a sense probe was used (data not shown).

Scale bar, 100 µm. Abbreviations are: cb, central body; lc, lateral calyx of the mushroom body; lo, lobula; me, medulla; mc, median calyx of the mushroom body; sog, subesophageal ganglion.

Figure 6: Western-blot analysis of protein fractions from honeybee-brain homogenates and Am5 HT_7-HA-expressing cell lines. **(A)** Western-blot analysis of proteins from honeybee-brain homogenates. Lane 1: Sample incubated with purified anti Am5-HT_7 antibody. One protein band of ~66 kDa was detected. This band most likely represents the native Am5-HT_7 receptor in its glycosylated state. Lane 2: Sample incubated with anti Am5-HT_7 antibody pre-incubated with MBP-CT fusion protein (the antigen used for immunization). No band is detected. **(B)** Western blot of membrane proteins from HEK 293 cells stably expressing Am5-HT_7 (cell lines 1 and 4) and of non-transfected HEK 293 cells (nt). The anti HA-tag antibody recognized a single protein band of ~70 kDa in line 1 and line 4 (lanes 2 and 4, respectively). After PNGase treatment, the apparent size of the protein (~56 kDa) agrees well with that deduced from the cloned Am*5-ht*7 cDNA (lanes 1 and 3). No bands were detected in protein preparations from non-transfected cells (lanes 5 and 6). **(C)** The identity of the heterologously expressed protein was confirmed by incubation of the blot (shown in **B**) with the specific anti Am5 HT_7 antibody.

Figure 7: Modulation of intracellular cAMP levels in HEK 293 cells stably expressing the Am5 HT_7-HA receptor (line 1 and line 4) and in non-transfected cells (nt). The cAMP produced is given as pmol cAMP/mg protein. Data are mean values of at least two independent experiments performed in duplicate. Error bars indicate SEM. **(A)** Effects of various biogenic amines and of the serotonin receptor agonist 5-CT on cAMP levels. To determine the basal $[cAMP]_i$, cells were incubated with 10 µM IBMX only. **(B)** Dose-

dependent effects of 5-HT on cAMP levels. Cells were incubated with increasing concentrations (10^{-10} to 10^{-5} M) of 5-HT. **(C)** Effects of the 5-HT receptor antagonists methiothepin and clozapine on 5-HT stimulated cAMP production. Antagonists were applied at a concentration of 10 μM in the presence of 10 μM 5-HT.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TM2 TM3
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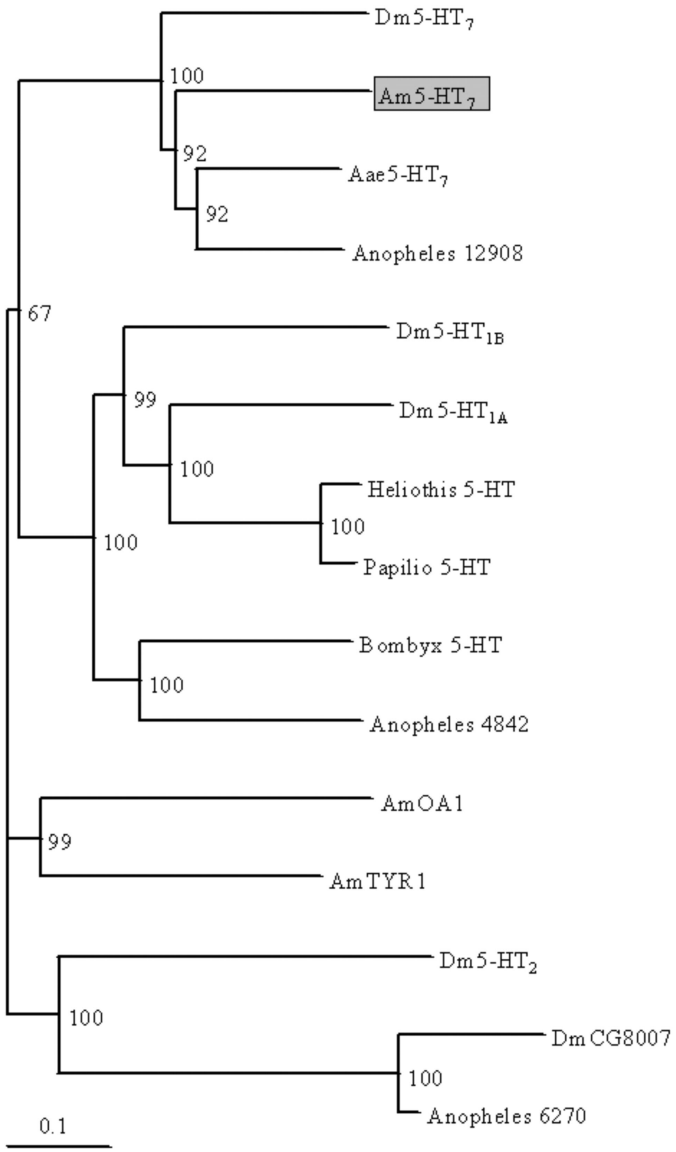
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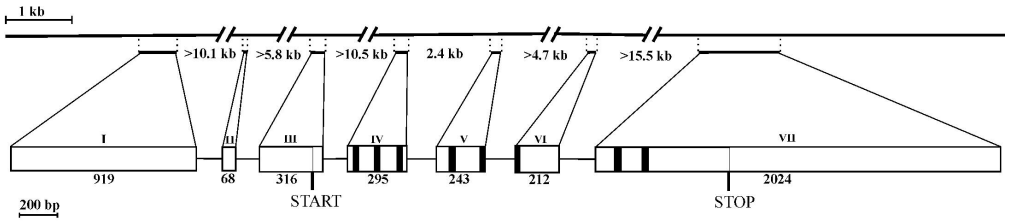
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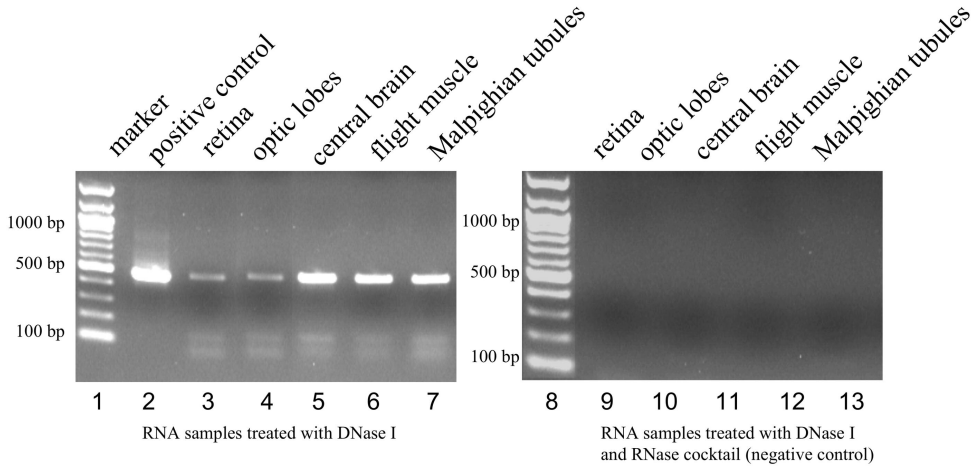
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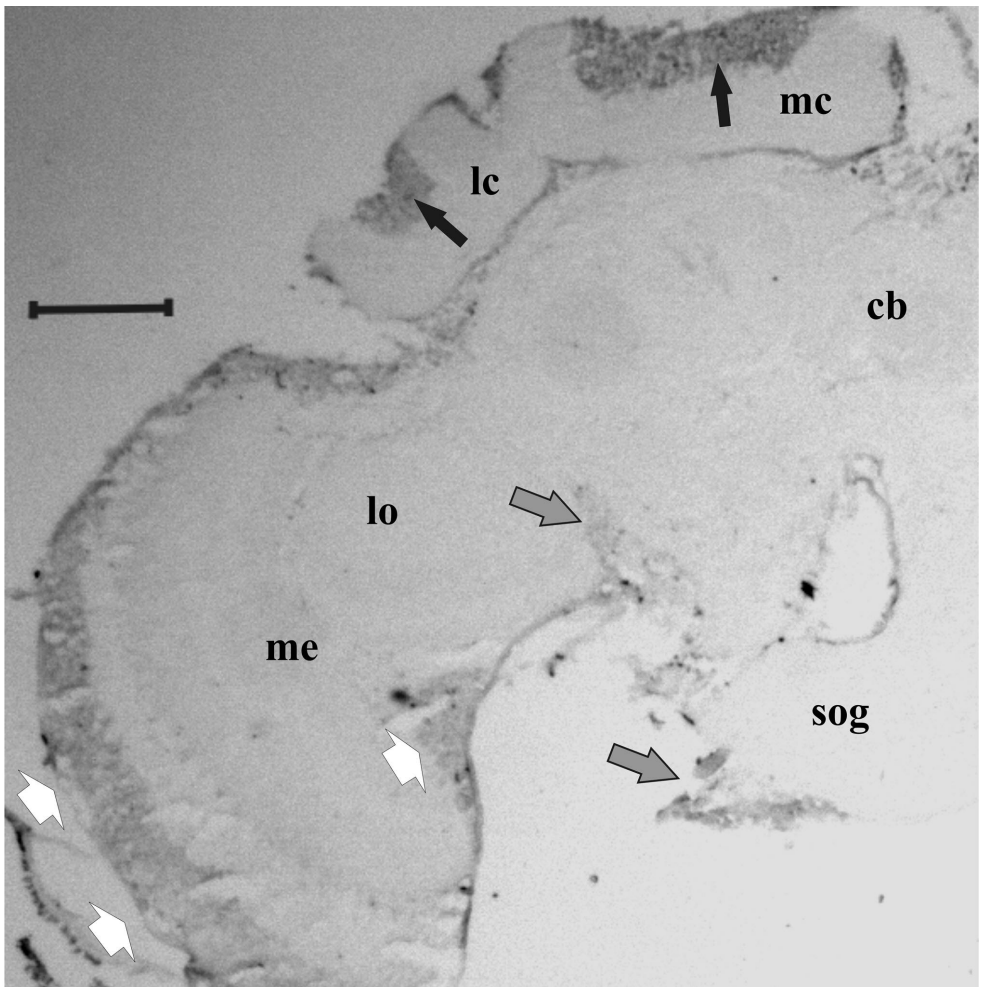
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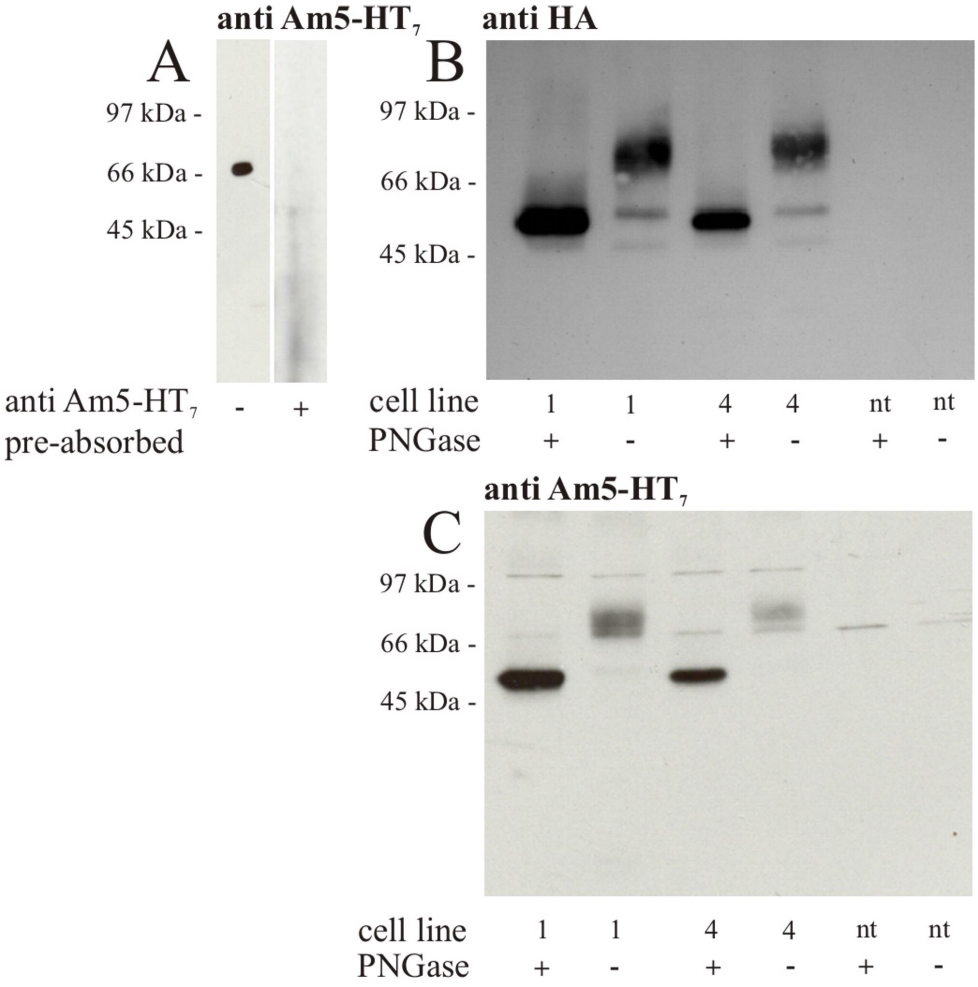
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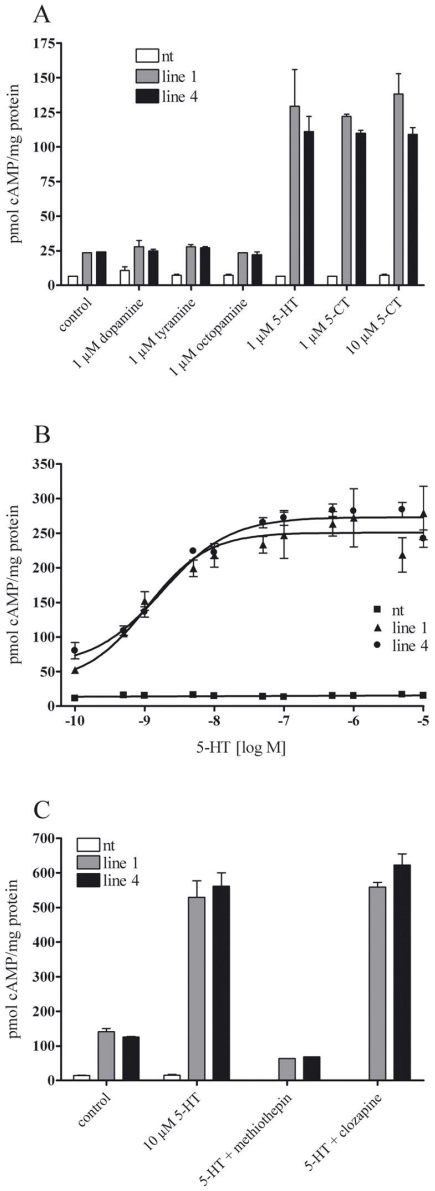
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10 µM 5-CT
B
pmol cAMP/mg protein
nt
line 1
line 4
5-HT [log M]
C
pmol cAMP/mg protein
nt
line 1
line 4
control
10 µM 5-HT
5-HT + methiothepin
5-HT + clozapine

Einfluss der biogenen Amine Dopamin, Tyramin und Octopamin auf gustatorische Reaktionsschwellen der Honigbiene

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Research report

Behavioural pharmacology of octopamine, tyramine and dopamine in honey bees☆

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Abstract

In the honey bee, responsiveness to sucrose correlates with many behavioural parameters such as age of first foraging, foraging role and learning. Sucrose responsiveness can be measured using the proboscis extension response (PER) by applying sucrose solutions of increasing concentrations to the antenna of a bee. We tested whether the biogenic amines octopamine, tyramine and dopamine, and the dopamine receptor agonist 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene (6,7-ADTN) can modulate sucrose responsiveness. The compounds were either injected into the thorax or fed in sucrose solution to compare different methods of application. Injection and feeding of tyramine or octopamine significantly increased sucrose responsiveness. Dopamine decreased sucrose responsiveness when injected into the thorax. Feeding of dopamine had no effect. Injection of 6,7-ADTN into the thorax and feeding of 6,7-ADTN reduced sucrose responsiveness significantly. These data demonstrate that sucrose responsiveness in honey bees can be modulated by biogenic amines, which has far reaching consequences for other types of behaviour in this insect.

Keywords: Honey bee; Insect; Proboscis extension response; Sucrose responsiveness; Biogenic amines

1. Introduction

Biogenic amines act as important neuromodulators, neurohormones and neurotransmitters in the honey bee (for a recent review see [3]). The biogenic amines octopamine and dopamine are found in high amounts in the central nervous system of adult honey bees [9,11,23,39]. Biogenic amines can act at all levels of the insect nervous and motor systems (for a recent review see [24]). They can modulate the sensitivity of sensory receptors and interneurons. But they can also modify the intrinsic properties of neurons, the efficacy of synaptic connections, and the properties of motor systems by acting on motoneurons and by directly affecting muscles. Consequently, the behavioural functions of biogenic amines are wide-ranging and concern reflex-like behaviours, sensory sensitivity, different forms of learning [24], and the formation of memory [20].

Recently, the cDNAs for several different biogenic amine receptors were cloned from the bee brain, heterologously expressed and functionally characterised. A receptor for dopamine (AmDOP1; [5]), a receptor for tyramine (AmTYR1; [2]) and an octopamine receptor (AmOCT1; [10]) were analysed in collaboration with our laboratory. The receptors are widely distributed in the brain and suboesophageal ganglion of the bee, suggesting involvement in different sensory and motor systems. We do not know whether the in-vitro characteristics of biogenic receptor ligands are also applicable to in-vivo conditions. The dopamine receptor agonist 6,7 ADTN,

Abbreviations: 6,7-ADTN, 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene; DOPA, dihydroxyphenylalanine; MI, modulation index; PER, proboscis extension response; PKA, protein kinase A; SRS, sucrose response score.

☆ Parts of this work have been published in abstract form [Proceedings of the 2001 Berlin Meeting of the European Sections of IUSSI (2001) 254].

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for example, is an effective agonist for the heterologously expressed dopamine D1 receptor AmDOP1 [5], but in contrast to dopamine it does not have an agonistic behavioural effect on the proboscis extension response (PER, see below) when injected into the α-lobe of the mushroom bodies of the bee [4]. Although there exist distinct receptors for tyramine and octopamine [2,10], the functional differences between these two biogenic amines are unclear. In the past, effects of tyramine application were usually attributed to octopamine, which is synthesised from tyramine [8].

To test the effects of amines in a behavioural assay we decided to analyse 'sucrose responsiveness' of the bee, because this behavioural parameter correlates with a number of different behaviours including reflex responses, different forms of learning, behavioural development and even the division of labour within the colony [24]. Sucrose responsiveness can be measured reliably using the PER. When the antenna of a bee is touched with a droplet of sucrose solution of sufficient concentration, the bee reflexively extends its proboscis. Sucrose responsiveness can be measured by applying sucrose solutions of increasing concentrations to the antennae. The lowest sucrose concentration which elicits proboscis extension is a measure of the individual sucrose response threshold of a bee [25].

Sucrose responsiveness has repeatedly been shown to determine associative proboscis extension learning [34–36]. Bees showing high sucrose responsiveness reach a higher level of acquisition and show less extinction than bees with low responsiveness. The difference between the individual response threshold for sucrose and the sucrose concentration used as reward determines the level of acquisition [34].

Division of foraging labour correlates directly with sucrose responsiveness. Pollen foragers are more responsive to sucrose than nectar foragers [25,27], regardless of their nutritional state. The sucrose responsiveness of 1-week-old bees determines whether a bee will later forage for nectar or pollen [28]. Bees with high sucrose responsiveness at 1-week-old are more likely to become pollen foragers, while bees with low responsiveness are more likely to become nectar foragers. Sucrose responsiveness also depends on the age of the bees. Newly emerged bees are not very responsive to sucrose. The older the bees become, the more responsive they become to sucrose stimuli. Foragers are the most responsive group of bees [27]. Sucrose responsiveness also correlates with non-associative habituation [32], responsiveness to pollen, and phototactic behaviour (J. Erber, unpublished results). Moreover, sucrose responsiveness correlates with the activity of protein kinase A in neural tissue [32]. These correlations make sucrose responsiveness an excellent indicator of the behavioural state of a bee. By measuring sucrose responsiveness one can make good predictions for different behaviours of a bee. So far, the complex mechanisms underlying the regulation and modulation of sucrose responsiveness are unknown.

Responsiveness to sucrose can be affected on different time scales. One factor which has a great influence on sucrose responsiveness is genotype. Bees of two genetic strains, which were selected for their pollen hoarding behaviour [26], differ systematically in their responsiveness. High-pollen-hoarding strain bees of all age groups are more responsive to sucrose than low-pollen-hoarding strain bees [25,27,30]. Environmental parameters such as humidity and air pressure affect responsiveness to water and sucrose on a shorter time scale (R. Scheiner, unpublished data). Parameters such as the amount of brood pheromone in the colony can modulate sucrose responsiveness on a similar time scale [29]. Short-term modulation depends on the degree of satiation, the concentration of the collected nectar and crop filling. Hungry bees are more responsive to sucrose than satiated bees [17,25,30]. Bees fed with low sucrose concentrations for 24 h are more responsive to sucrose than bees fed with a high sucrose concentration for the same time [30]. Bees are more responsive to sucrose before consuming water for some seconds, although the water does not have any nutritional value [30].

The aim of the study presented here was to analyse the biogenic amines octopamine, tyramine and dopamine, and the dopamine receptor agonist 6,7-ADTN [4] for their effects on sucrose responsiveness. We also wanted to compare different methods of application under standardised conditions. Therefore, we applied the compounds either by injection into the haemolymph or by feeding. As sucrose responsiveness correlates with diverse types of behaviour in the bee, these experiments will also help us to understand the functions of biogenic amines for these behaviours in this insect.

2. Materials and methods

2.1. Measuring of sucrose responsiveness

Sucrose responsiveness was measured using the PER. The antennae of each bee were touched with a droplet of water or sucrose solution of increasing concentrations and it was recorded whether the bee extended its proboscis. The following sucrose concentrations were applied in ascending order 0.1, 0.3, 1, 3, 10 and 30%. Sucrose responsiveness in different bees shows a great variation in this range of concentrations [25,37]. This variation in responsiveness is a sensitive measure of the behavioural state of the bees. Prior to each sucrose solution the bees were stimulated with water to avoid sensitisation. The inter-stimulus interval was approximately 2 min.

As a measure for sucrose responsiveness we calculated the 'sucrose response score'. The sucrose response score

of a bee is the total number of the bee's responses to all sucrose stimulations of increasing concentration [33–35]. With the six different sucrose concentrations used in our experiments the sucrose response score can vary between zero (an animal that did not respond to sucrose at all) and six (an animal that responded to all sucrose stimuli).

2.2. Injections into the thorax

All neuroactive substances used for injections or feeding were purchased from Sigma. For the injections into the thorax returning forager bees which did not carry pollen loads were individually caught at the hive entrance, immobilised at 4 °C in the refrigerator and mounted in small metal tubes with strips of adhesive tape between head and thorax to secure the bee. A second strip of adhesive tape was fixed over the abdomen. The heads of the bees were fixed to the tube with a mixture of bee wax and colophony. The bees remained in the tubes till the start of the behavioural experiment (ca. 14 h). The fixation of the head is necessary to reduce movements of the animal during injection and does not affect sucrose responsiveness (R. Scheiner, personal observation). Bees were fed to repletion with 30% sucrose the evening before the experiments started. Two hours prior to the experiment each bee was fed again with three droplets of 30% sucrose solution, which approximately amounts to 15 μl. From previous experiments we know that this feeding results in an intermediate satiation status of the animals for the time of the experiments. Initial responsiveness to sucrose, which serves as a reference for the injection experiments, was measured in each bee as described above.

Then, 1 μl of the neuroactive substances shown in Table 1 dissolved in saline solution which contained 10^{-2} M ascorbic acid was injected into the thorax of each bee. Saline (270 mM NaCl, 3.2 mM KCl, 1.2 mM $CaCl_2$, 10 mM MgCl, 10 mM morpholinopropansulfonic acid, pH 7.4) containing 10^{-2} M ascorbic acid was injected as a control. As common in experiments with biogenic amines [6], ascorbic acid was added to the solution to minimize oxidation of the biogenic amines.

Sucrose responsiveness was tested again 30 min after the injection. A number of earlier experiments have shown that the effects of transmitters reach a stable level approximately 30 min after drug application [4,19,21]. The neuroactive substances were injected in fairly high concentrations, because considerable dilution will occur before the compounds reach their targets in the brain. The different amounts of the neuroactive substances were chosen to test for dose effects.

As sucrose responsiveness in groups of bees can vary from day to day depending on weather and intracolonial conditions, it is important to measure the respective control animals at approximately the same time as the experimental groups. We, therefore, measured behaviour in the control groups on the same days as the experimental groups. Variations between individuals were compensated for by comparing the same animals before and after treatment. The number of bees tested is shown in Table 1.

2.3. Feeding experiments

For this experiment forager bees that returned to the hive without a pollen load were individually captured at the hive entrance, cooled to immobilisation in a refrigerator maintained at 4 °C and mounted as described above. The heads of the bees were not fixed to the tube and the bees were not fed sucrose before the start of the experiment. The bees remained in the tubes for 1/2 h before initial sucrose responsiveness was measured as described above. Directly after measuring responsiveness each individual was either fed with 10 μl of a 30% sucrose solution containing ascorbic acid or with one of the neuroactive substances dissolved in 30% sucrose solution containing ascorbic acid (see Table 1). As in the injection experiment, sucrose responsiveness was measured again 30 min after drug application. The number of bees tested is shown in Table 1.

2.4. Statistics

For statistical analysis and graphic display, a modulation index (MI) was calculated. This index is a sensitive measure of changes in sucrose responsiveness.

$$\mathrm{MI}(t) = \frac{\mathrm{SRS}_t - \mathrm{SRS}t_0}{\mathrm{SRS}_t + \mathrm{SRS}t_0}$$

with $\mathrm{SRS}t_0$, sucrose response score before injection or feeding; SRS_t, sucrose response score at time t after injection or feeding.

The MI can range between -1 and $+1$. A positive index marks an increase in sucrose responsiveness. A negative index implies a decrease in responsiveness. An index of 0 indicates that sucrose responsiveness in an individual did not change after treatment. As the MI of bees which had a response score of 0 before and after treatment is not defined, these animals also received a MI of 0. The modulation indices of different groups were not always normally distributed. Therefore, median values and quartiles for the modulation indices are shown in the figures. Different treatment groups were compared using two-tailed non-parametric Mann–Whitney U-tests.

Table 1
Absolute amounts of injected neuroactive compounds, concentrations in the injected volumes, and number of bees tested in the different experiments

Substance	Form of application and volume	Absolute amount of injected compound, concentration in the injected volume and number of bees tested
Octopamine	Injection into thorax (1 μl)	0.0 μg (saline): $n = 40$
		0.19 μg (10^{-3} M): $n = 42$
		1.9 μg (10^{-2} M): $n = 42$
	Feeding (10 μl)	0.0 μg (sucrose): $n = 38$
		0.19 μg (10^{-4} M): $n = 35$
		1.9 μg (10^{-3} M): $n = 37$
		19.0 μg (10^{-2} M): $n = 37$
Tyramine	Injection into thorax (1 μl)	0.0 μg (saline): $n = 40$
		0.17 μg (10^{-3} M): $n = 43$
		1.7 μg (10^{-2} M): $n = 42$
	Feeding (10 μl)	0.0 μg (sucrose): $n = 74$
		1.7 ng (10^{-6} M): $n = 38$
		17.0 ng (10^{-5} M): $n = 38$
		0.17 μg (10^{-4} M): $n = 74$
		1.7 μg (10^{-3} M): $n = 34$
		17.0 μg (10^{-2} M): $n = 37$
Dopamine	Injection into thorax (1 μl)	0.0 μg (saline): $n = 41$
		0.19 μg (10^{-3} M): $n = 41$
		1.9 μg (10^{-2} M): $n = 41$
		19.0 μg (10^{-1} M): $n = 41$
	Feeding (10 μl)	0.0 μg (sucrose): $n = 39$
		0.19 μg (10^{-4} M): $n = 39$
		1.9 μg (10^{-3} M): $n = 38$
		19.0 μg (10^{-2} M): $n = 38$
6,7-ADTN	Injection into thorax (1 μl)	0.0 μg (saline): $n = 41$
		26.0 ng (10^{-4} M): $n = 41$
		0.26 μg (10^{-3} M): $n = 41$
		2.6 μg (10^{-2} M): $n = 41$
	Feeding (10 μl)	0.0 μg (sucrose): $n = 47$
		0.26 μg (10^{-4} M): $n = 46$
		2.6 μg (10^{-3} M): $n = 51$
		26.0 μg (10^{-2} M): $n = 52$

3. Results

3.1. Effects of octopamine on sucrose responsiveness

Injection of octopamine into the thorax of honey bees significantly increased their responsiveness to sucrose (Fig. 1A). The modulation indices of bees which had been injected with 0.19 μg octopamine or with 1.9 μg octopamine were significantly higher than was the index of the controls (0.19 μg: $n = 42$, $P \leq 0.001$, 1.9 μg: $n = 42$, $P \leq 0.05$, $n_{controls} = 40$; two-tailed Mann–Whitney U-test). The two different octopamine concentrations did not lead to significantly different modulation indices ($P > 0.05$, two-tailed Mann–Whitney U-test).

Feeding of octopamine also increased sucrose responsiveness significantly (Fig. 1B). The modulation indices of bees treated with 1.9 μg octopamine and those of bees fed with 19 μg octopamine were significantly higher than was the index of the sucrose-fed controls (1.9 μg: $n = 37$, $P \leq 0.05$, 19 μg: $n = 37$, $P \leq 0.05$, $n_{controls} = 38$; two-tailed Mann–Whitney U-test). The lower octopamine concentration did not affect sucrose responsiveness (0.19 μg: $n = 35$, $P > 0.05$; two-tailed Mann–Whitney U-test). The modulation indices of the bees fed with the two octopamine doses which resulted in a change of sucrose responsiveness did not differ significantly between each other ($P > 0.05$; two-tailed Mann–Whitney U-test).

3.2. Effects of tyramine on sucrose responsiveness

Injection of tyramine into the thorax of honey bees significantly increased their responsiveness to sucrose (Fig. 2A). Bees injected with 0.17 μg tyramine or with 1.7 μg tyramine showed a significantly higher MI 30 min after injection than controls (0.17 μg: $n = 43$, $P \leq 0.05$, 1.7 μg: $n = 42$, $P \leq 0.05$, $n_{controls} = 40$; two-tailed Mann–Whitney U-test). The two tyramine concentrations did not lead to significantly different modulation indices ($P > 0.05$, two-tailed Mann–Whitney U-test).

Feeding of tyramine also increased sucrose responsiveness significantly (Fig. 2B). The modulation indices of bees fed with 0.17 μg tyramine or with 17 μg tyramine were significantly higher than was the index of sucrose-fed control bees (0.17 μg: $n = 74$, $P \leq 0.001$, 17 μg: $n =$

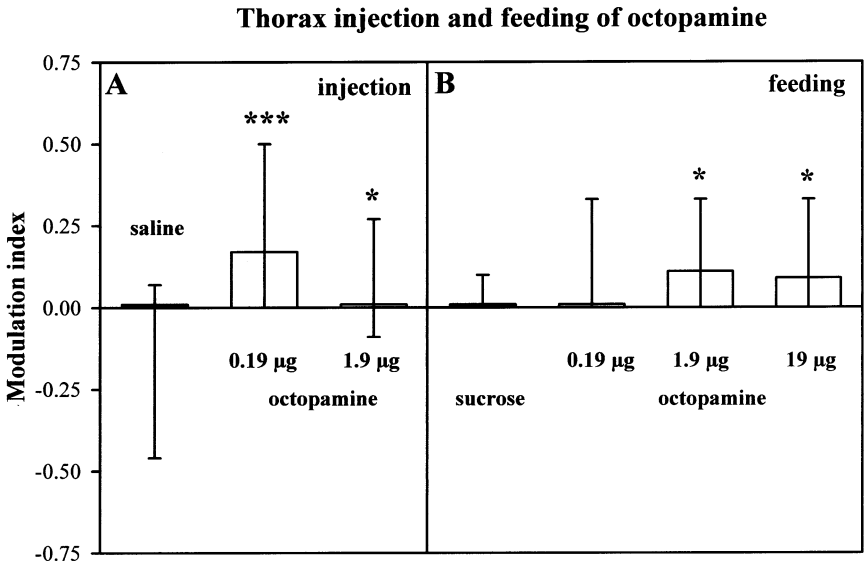

Fig. 1. Modulation of sucrose responsiveness by injecting octopamine into the thorax (A) or by feeding (B) octopamine in a 30% sucrose solution. Median modulation indices and quartiles are shown. The amounts of applied octopamine are given. Both injection into the thorax and feeding of octopamine lead to a significant increase in sucrose responsiveness. Significant differences between the treated groups and the controls are indicated by asterisks. *$P \leq 0.05$; ***$P \leq 0.001$, two-tailed Mann–Whitney U-test.

37, $P = 0.01$, $n_{controls} = 74$; two-tailed Mann–Whitney U-test). The lower tyramine concentrations did not lead to significant effects (1.7 ng: $n = 38$, $P > 0.05$, 17 ng: $n = 38$, $P > 0.05$; two-tailed Mann–Whitney U-test). Bees fed with the amounts of tyramine which led to a significant change in responsiveness did not differ in their modulation indices between each other ($P > 0.05$; two-tailed Mann–Whitney U-test).

3.3. Effects of dopamine on sucrose responsiveness

Injection of dopamine into the thorax significantly decreased responsiveness to sucrose (Fig. 3A). The modulation indices of bees injected with 1.9 µg dopamine or with 19 µg dopamine were significantly lower 30 min after treatment than was the index of saline-injected controls (1.9 µg: $n = 41$, $P \leq 0.05$, 19 µg: $n = 41$, $P \leq 0.05$, $n_{controls} = 41$; two-tailed Mann–Whitney U-test).

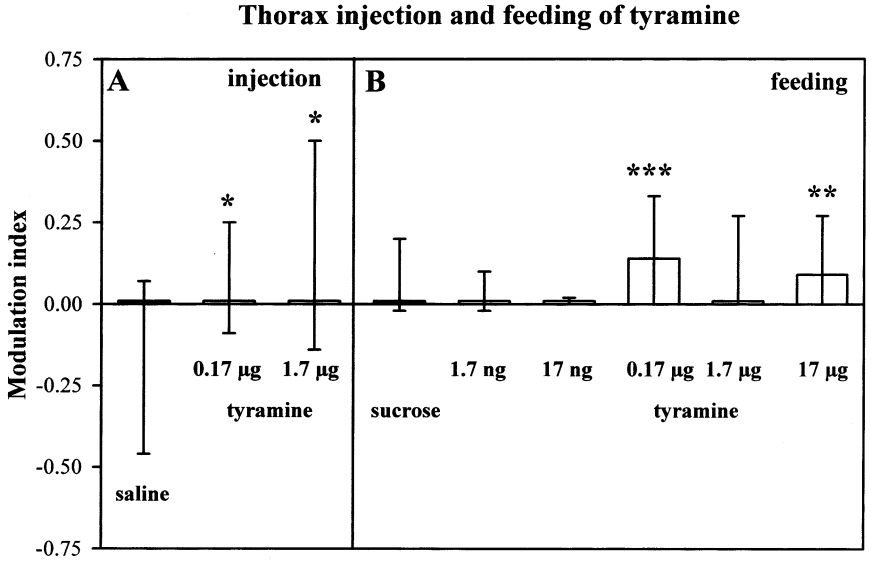

Fig. 2. Modulation of sucrose responsiveness by injecting tyramine into the thorax (A) or by feeding (B) tyramine in a 30% sucrose solution. Median modulation indices and quartiles are shown. The amounts of applied tyramine are given. Both injection of different amounts of tyramine and feeding of different amounts of tyramine increase sucrose responsiveness. Significant differences between the treated groups and the controls are indicated by asterisks. *$P \leq 0.05$; **$P \leq 0.01$, ***$P \leq 0.001$; two-tailed Mann–Whitney U-test.

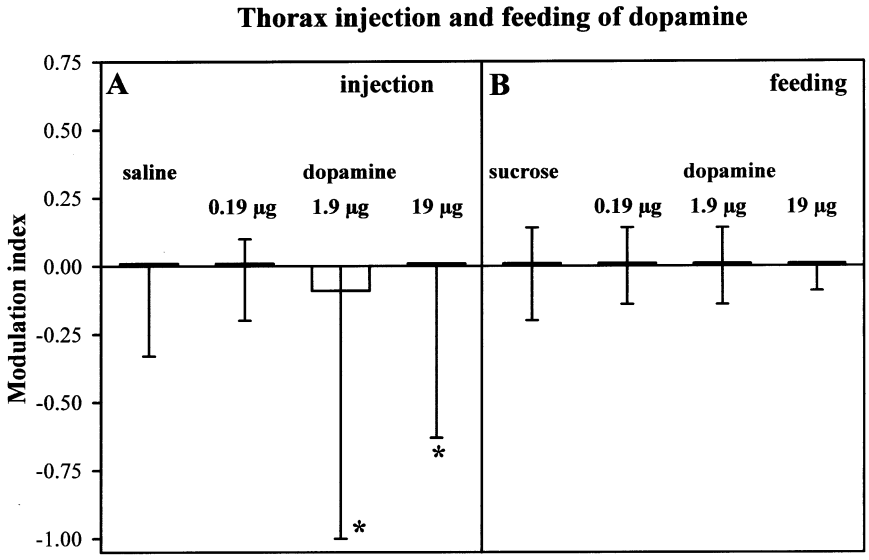

Fig. 3. Modulation of sucrose responsiveness by injecting dopamine into the thorax (A) or by feeding (B) dopamine in a 30% sucrose solution. Median modulation indices and quartiles are shown. The amounts of applied dopamine are given. Whereas injection of different amounts of dopamine leads to a significant decrease in sucrose responsiveness, feeding of dopamine does not affect responsiveness. Significant differences between the treated groups and the controls are indicated by asterisks. *$P \leq 0.05$, two-tailed Mann–Whitney U-test.

The lower dopamine concentration did not affect sucrose responsiveness (0.19 µg: $n = 41$, $P > 0.05$; two-tailed Mann–Whitney U-test).

Feeding of dopamine did not affect sucrose responsiveness (Fig. 3B). The MI of bees fed with sucrose did not differ from the indices of bees fed with different amounts of dopamine (0.19 µg: $n = 39$, $P > 0.05$, 1.9 µg: $n = 38$, $P > 0.05$, 19 µg: $n = 38$, $P > 0.05$, $n_{controls} = 39$; two-tailed Mann–Whitney U-test).

3.4. Effects of 6,7-ADTN on sucrose responsiveness

Injection of the dopamine receptor agonist 6,7-ADTN into the thorax decreased responsiveness to sucrose significantly (Fig. 4A). Bees which had been injected with 26 ng or with 0.26 µg of 6,7-ADTN had significantly lower modulation indices than had saline controls (0.026 µg: $n = 41$, $P = 0.01$, 0.26 µg: $n = 41$, $P = 0.05$, $n_{controls} = 41$; two-tailed Mann–Whitney U-test). Interestingly, the highest 6,7-ADTN concentration did not affect sucrose responsiveness (2.6 µg: $n = 41$, $P > 0.05$; two-tailed Mann–Whitney U-test). The bees treated with the two 6,7-ADTN amounts which affected sucrose responsiveness did not differ in their modulation indices between each other ($P > 0.05$; two-tailed Mann–Whitney U-test).

In contrast to dopamine, feeding of 6,7-ADTN did affect sucrose responsiveness (Fig. 4B). The modulation indices of bees which had been fed with 2.6 µg 6,7-ADTN or with 26 µg 6,7-ADTN were significantly lower than was the MI of sucrose-fed control bees (2.6 µg: $n = 51$, $P = 0.01$, 26 µg: $n = 52$, $P = 0.05$, $n_{controls} = 47$; two-tailed Mann–Whitney U-test). Only the lowest 6,7-ADTN concentration did not lead to significant effects (0.26 µg: $n = 46$, $P > 0.05$; two-tailed Mann–Whitney U-test). The modulation indices of the bees which were treated with the two 6,7-ADTN concentrations affecting sucrose responsiveness did not differ between each other ($P > 0.05$; two-tailed Mann–Whitney U-test).

4. Discussion

4.1. Octopamine and tyramine increase sucrose responsiveness

Injection and feeding of octopamine or tyramine significantly increased sucrose responsiveness in honey bees. These results support earlier findings demonstrating that octopamine and tyramine can increase the rate of proboscis extension in honey bees stimulated with high concentrations of sucrose [8,20]. As only high sucrose concentrations were used in those earlier experiments, it was impossible to determine the modulatory effects of these amines on responsiveness to sucrose. Even bees with low sucrose responsiveness will respond to high sucrose concentrations [25,34–36] which makes it very difficult to detect differences in sucrose responsiveness following the application of biogenic amines.

A number of experiments demonstrate that octopamine enhances responsiveness in unresponsive animals [24]. Injection of octopamine restores the PER in fully satiated bees, which did not show proboscis extension prior to injection [20]. Octopamine injections into the antennal lobe stimulate feeding behaviour [1]. Reser-

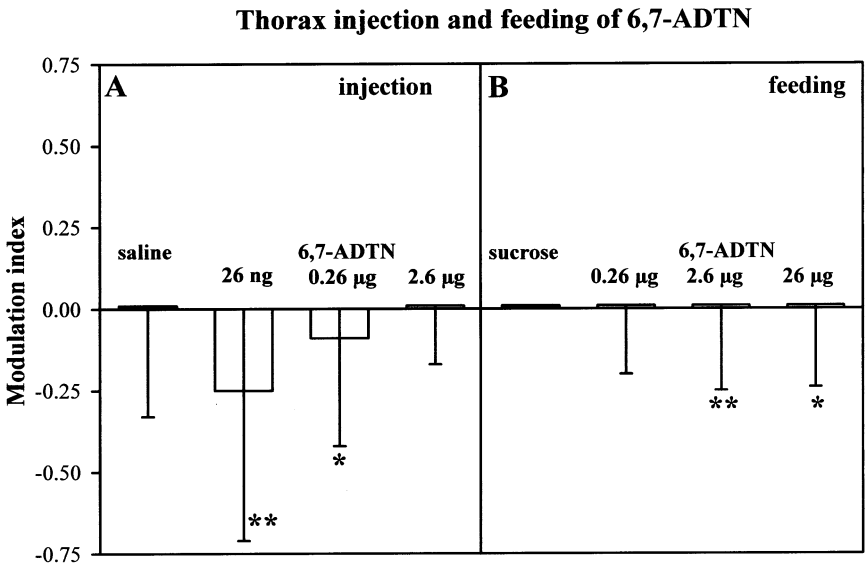

Fig. 4. Modulation of sucrose responsiveness by injecting 6,7-ADTN into the thorax (A) or by feeding (B) 6,7-ADTN in a 30% sucrose solution. Median modulation indices and quartiles are shown. The amounts of applied 6,7-ADTN are given. Both injection of different amounts of 6,7-ADTN and feeding of different amounts of 6,7-ADTN lead to a significant decrease in sucrose responsiveness. Significant differences between the treated groups and the controls are indicated by asterisks. *$P \leq 0.05$; **$P \leq 0.01$, two-tailed Mann–Whitney U-test.

pine, which inhibits the vesicular uptake of biogenic amines, depletes the nervous system of monoamines. In an experiment by Braun and Bicker [8] 30% of the reserpinised bees showed no longer the PER, the rest displayed lower response amplitudes than controls. Injection of tyramine or octopamine into the haemolymph of the reserpinised bees restored the PER. A stimulating effect of octopaminergic agonists on feeding behaviour was also demonstrated for the blowfly [15]. Taken together, all these experiments demonstrate that an increase in responsiveness of the sucrose system is a common effect of exogenously applied octopamine and tyramine. It has to be determined whether the applied tyramine is metabolised in vivo to octopamine or directly acts by binding to specific tyramine or octopamine receptors.

4.2. Dopamine and 6,7-ADTN decrease sucrose responsiveness

Injection of dopamine or the dopamine receptor agonist 6,7-ADTN decreased sucrose responsiveness, with 6,7-ADTN being more potent than dopamine. Whereas injection of 26 ng 6,7-ADTN into the thorax led to a significant decrease in responsiveness to sucrose, only a much higher dose of dopamine (1.9 µg) had a similar effect. Feeding of 6,7-ADTN significantly decreased responsiveness to sucrose, while feeding of dopamine had no effect. These findings suggest that 6,7-ADTN is acting on one or both of the D1-like dopamine receptors, AmDOP1 [4] and AmDOP2 (J.A. Mustard, W. Blenau, V.K. Ward, P.R. Ebert and A.R. Mercer, unpublished data), because 6,7-ADTN was shown to be a powerful agonist for these receptors. Earlier experiments in which responsiveness to water vapour was measured did not show effects of 6,7-ADTN when injected into the α-lobe of the mushroom bodies [4]. Our present experiments demonstrate that 6,7-ADTN can reduce sucrose responsiveness when fed or injected into the thorax.

There are only few studies on the effects of dopamine or its receptor agonists on the elicitation of sucrose-induced proboscis extension. Mercer and Menzel [22] and Macmillan and Mercer [16] showed that dopamine injection into different brain areas did not affect the elicitation of sucrose-induced PER. But the sucrose concentrations used were probably too high to detect differences in sucrose responsiveness (see above). Whereas octopamine and tyramine restored the PER in reserpinised bees, dopamine had no effect on PER [8]. On the basis of our experiments, we assume that reserpine treatment reduced sucrose responsiveness to a minimum. Therefore, an additional reduction of responsiveness by dopamine should not be detected.

4.3. Mode of application

Our experiments allow to compare the different modes of application for the neuroactive substances under standardised conditions using the same stimuli and behavioural measure. In a number of earlier experiments with bees the effects of biogenic amines were tested using different types of behaviour and different modes of application, which sometimes led to contradictory results. Our results clearly demonstrate that all compounds tested act on sucrose responsiveness

when injected into the haemolymph. In contrast to injection into the thorax, feeding of the neuroactive substances in a sucrose solution was not always effective and the necessary amounts of transmitters to cause an effect were often higher than in the injection experiments.

It is difficult to interpret the differences between injection and feeding of the compounds, because very little is known about the metabolic processes acting on the substances after oral uptake and after injection. Injection of biogenic amines into the haemolymph of honey bees has been used successfully for many years e.g. [31] and it was shown in the cabbage looper moth, *Trichoplusia ni*, that injections increase brain levels of biogenic amines [14]. Feeding octopamine to bees also results in a significant increase in brain levels of octopamine [38]. At the moment, we cannot unequivocally attribute the effects to specific compounds. Even measuring the amount of transmitters or their metabolites in the brain after the different treatments does not help to identify the compounds, which induce the behavioural changes.

Apart from dopamine, all substances tested changed sucrose responsiveness both after injection and after feeding. In the case of dopamine we assume that after feeding only small amounts of this transmitter reached the structures controlling sucrose responsiveness. This might be due to rapid oxidation of dopamine, which prevents short-term behavioural effects. Dopamine possibly only acts on behaviour when fed for a long time, which is supported by a finding of Božič and Woodring [7] showing that feeding ad libitum on a sucrose solution containing the dopamine precursor DOPA (dihydroxyphenylalanine) significantly increased the rate of dancing in foragers.

4.4. Biogenic amines, sucrose responsiveness and behaviour

Our findings collectively suggest that the biogenic amines octopamine, tyramine and dopamine are involved in the regulation and modulation of sucrose responsiveness. But sucrose responsiveness may also be affected by other parameters such as the expression of biogenic amine receptors [13], the amount of released transmitter, the synaptic efficacy at specific target sites and the intrinsic properties of the cells involved in controlling sucrose responsiveness. It is, therefore, not astonishing that the results of experiments analysing biogenic amine levels and behaviour in bees are sometimes contradictory. Foragers, for example, have higher levels of octopamine in the central brain than bees tending the larvae in the hive [39], and foragers are more responsive to sucrose than younger hive bees [27]. This higher level of octopamine could explain why these bees are more responsive to sucrose. But at the same time foragers also have higher levels of dopamine than younger bees [39], which should make them less responsive to sucrose. Schulz and Robinson [38] showed that feeding of octopamine on a longer time scale reduces the age of first foraging, possibly via the increased release of juvenile hormone from the corpora allata [12]. These results correlate with the experimental finding that high sucrose responsiveness of young bees correlates with an early age of first foraging [24]. But at the moment it is unclear whether the octopamine level is the only factor contributing to these effects.

In addition to the division of labour, biogenic amines have been shown to modify a number of different behaviours such as sensory sensitivity, reflex responses, motor responses, and different forms of learning [24]. Octopamine, for example, restores proboscis extension conditioning in reserpinised bees, whereas dopamine does not [18]. Some of these behaviours, such as PER learning, have been demonstrated to correlate with sucrose responsiveness [32,34–36]. Bees with a high sucrose responsiveness show a higher PER learning rate than bees with low sucrose responsiveness. In our view, the biogenic amines octopamine, tyramine and dopamine affect certain forms of behaviour by increasing or decreasing behavioural thresholds. Octopamine and tyramine, for example, should improve proboscis extension learning, because the amines increase responsiveness to the unconditioned sucrose stimulus. Dopamine should not improve learning performance, because it decreases responsiveness to the unconditioned sucrose stimulus. We assume that biogenic amines also affect the division of labour by changing response-releasing thresholds. We think that our experiments establish a causal link between biogenic amine levels, responsiveness to sucrose stimuli, and different types of behaviour.

Acknowledgements

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Die Speicheldrüsen der Schabe *Periplaneta americana* als Modellobjekt zur Untersuchung der zellulären Wirkungen biogener Amine

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The Aminergic Control of Cockroach Salivary Glands

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The acinar salivary glands of cockroaches receive a dual innervation from the subesophageal ganglion and the stomatogastric nervous system. Acinar cells are surrounded by a plexus of dopaminergic and serotonergic varicose fibers. In addition, serotonergic terminals lie deep in the extracellular spaces between acinar cells. Excitation-secretion coupling in cockroach salivary glands is stimulated by both dopamine and serotonin. These monoamines cause increases in the intracellular concentrations of cAMP and Ca^{2+}. Stimulation of the glands by serotonin results in the production of a protein-rich saliva, whereas stimulation by dopamine results in saliva that is protein-free. Thus, two elementary secretory processes, namely electrolyte/water secretion and protein secretion, are triggered by different aminergic transmitters. Because of its simplicity and experimental accessibility, cockroach salivary glands have been used extensively as a model system to study the cellular actions of biogenic amines and to examine the pharmacological properties of biogenic amine receptors. In this review, we summarize current knowledge concerning the aminergic control of cockroach salivary glands and discuss our efforts to characterize *Periplaneta* biogenic amine receptors molecularly. Arch Insect Biochem Physiol 62:000–000, 2006.

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KEYWORDS:

INTRODUCTION

Biogenic amines act as neurotransmitters, neurohormones, or neuromodulators in the nervous system and in various peripheral organs of vertebrates and invertebrates (for reviews, see: Evans, 1980; Roeder, 1994; Blenau and Baumann, 2001; Baumann et al., 2003). Full comprehension of the complex physiological actions of biogenic amines in insects requires detailed knowledge about the molecular identity of the corresponding receptor proteins, their pharmacological properties, their tissue distribution, and the molecular mechanisms that link receptor activation to the various cellular responses.

Salivary glands of cockroaches are favorable objects for studying the actions of biogenic amines in insects as their secretory activity is controlled by these substances (House, 1980; House and Ginsborg, 1985; Ali, 1997; Zimmermann and Walz, 2003). The use of non-invasive optical methods and molecular methods is beginning to unravel the physiology of insect salivary glands and the cellular actions of biogenic amines.

In this review, we supplement the existing literature (House, 1980; House and Ginsborg, 1985) by summarizing recent advances and draw attention to some open questions about the aminergic control of cockroach salivary glands. This preparation permits the dissection of cellular processes induced by either dopamine (DA) or serotonin (5-hydroxytryptamine, 5-HT). Elementary processes of amine-induced saliva production and the modification of the primary saliva are performed by

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separate cell types that can be stimulated by either DA, 5-HT, or by both neurotransmitters.

MORPHOLOGY

The salivary glands of insects have either a tubular or an acinar organization; those of Dipteran flies (*Calliphora vicina, Drosophila melanogaster*) possess tubular glands, whereas cockroaches and locusts have acinar-type salivary glands. In the cockroaches *Periplaneta americana* and *Nauphoeta cinerea*, the salivary glands proper are part of a larger salivary gland complex (Fig. 1a). The paired acinar glands flank the foregut and are associated with a pair of sac-like reservoirs. Ductules emanate at the secretory acini and converge into a pair of salivary ducts that run in parallel and close to the reservoir ducts. The salivary ducts then fuse to give the main salivary duct. The reservoir ducts similarly fuse to give the main reservoir duct. The main salivary duct then enters into the main reservoir duct, which finally opens into the hypopharynx (Kessel and Beams, 1963; House and Ginsborg, 1985).

Secretory acini in the cockroach salivary gland have a uniform structural layout (Just and Walz, 1994a). They consist of three cell types: a pair of pyramidal peripheral cells (p-cells) at the base of each acinus, approximately eight central cells (c-cells) that are arranged around the acinar lumen, and flat fenestrated centroacinar cells that line the acinar lumen (Fig. 1b). Ultrastructural examination indicated early on that the three cell types have different functions. The p-cells have an extensive basal labyrinth, numerous mitochondria, and long microvilli that extend into the acinar lumen, suggesting that these cells are involved in electrolyte and water transport (Kessel and Beams, 1963; Sutherland and Chillseyzn, 1968). The c-cells, which contain a large number of secretory granules, supply the proteinaceous components to the saliva (Just and Walz, 1994a, 1996). The centroacinar cells are not thought to be directly involved in saliva formation but rather secrete a thin fenestrated cuticular intima toward their luminal surface.

Duct cells distal to the acini seem to be specialized for ion (and water?) transport because they

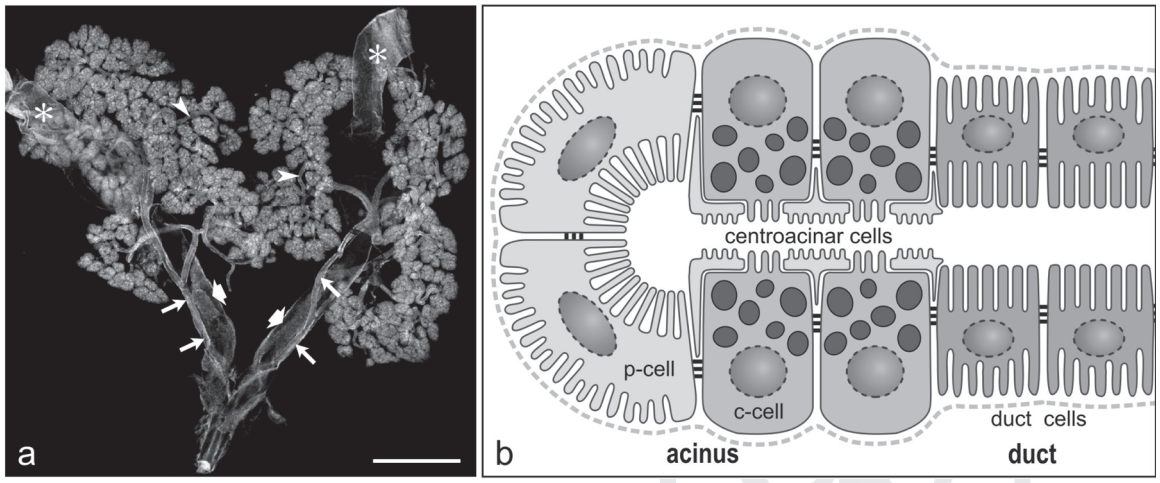

Fig. 1. Morphology of the salivary gland in the cockroach *Periplaneta americana*. **a:** Low-power micrograph of the salivary gland complex. The paired salivary glands consist of several lobes of acinar tissue. The ductules (arrowheads) that emerge from the acinar tissue unite to a single salivary duct (arrows) for each gland. The paired reservoirs (asterisks) open into reservoir ducts (broad arrows). **b:** Schematic representation of an acinus. Each acinus comprises two peripheral cells (p-cells) with long microvilli, approximately eight central cells (c-cells) with numerous secretory granules, and centroacinar cells that plaster the luminal surface of the c-cells. The duct cells have deep infoldings on both their basal and apical sides. Septate junctions (triple black lines) connect adjacent cells. Bar = 2 mm. Modified from Baumann et al. (2002). AQ5

have a prominent basal labyrinth, numerous mitochondria, and a highly folded apical (luminal) surface. On its cytoplasmic side, the entire apical membrane is coated with electron-dense 10-nm particles (Just and Walz, 1994a), representing parts of vacuolar-type H^+-ATPase molecules (see below, and: Just and Walz, 1994b; Zimmermann et al., 2003). Apically, the duct cells are covered by a thin continuous cuticular intima. The duct system of the salivary gland is extensive, thus raising the possibility that structural and functional properties vary along the tubules. Indeed, the duct cells located immediately proximal to the acini have been shown to contain secretory granules in *Nauphoeta cinerea* but not in *Periplaneta americana* (House and Ginsborg, 1985; Just and Walz, 1994a).

INNERVATION

Cockroach salivary glands receive innervation from two sources, the subesophageal ganglion and the stomatogastric nervous system (Willey, 1961; Davis, 1985; House and Ginsborg, 1985; Gifford et al., 1991; Elia et al., 1994; Ali, 1997). Two paired salivary neurons (SN1 and SN2), with their somata located in the subesophageal ganglion, send their axons contralaterally (SN1) or ipsilaterally (SN2) toward the salivary duct nerves (nerve 7b). SN1 contains DA and seems to be the only source of dopaminergic innervation of the salivary gland (Elia et al., 1994; Baumann et al., 2002). The neurotransmitter of SN2 has not been identified unequivocally in cockroaches. Rather than 5-HT, as in SN2 of *Locusta* (Ali et al., 1993; Ali, 1997), SN2 neurons of *Periplaneta* seem to contain γ-aminobutyric acid (GABA; O. Baumann, C. Hinnerichs, P. Dames, unpublished results) similar to SN2 in *Schistocerca* (Watkins and Burrows, 1989). Each salivary duct nerve in *Periplaneta* contains two axons with a diameter of ~5–7 μm. One of them is derived from SN1 (= dopaminergic) and the other from SN2 (probably GABAergic). In addition, several axons of smaller diameter are located peripherally within the salivary duct nerve (Whitehead, 1971). These fibers are serotonergic and form a neurohemal structure in the salivary duct nerve but also project onto the salivary glands proper (Davis, 1985; Baumann et al., 2002). A second source of serotonergic innervation of the salivary gland is via paired salivary nerves that originate from the esophageal nerve of the stomatogastric nervous system (Willey, 1961; Baumann et al., 2002).

The spatial distribution of dopaminergic and serotonergic nerve fibers and their synapses at the various cell types in the salivary gland complex of *Periplaneta* has recently been studied in detail (Baumann et al., 2002, 2004). The acinar tissue is entangled in a meshwork of serotonergic and dopaminergic varicose fibers. Dopaminergic and serotonergic fibers on the outer surface of the acini have release sites that are separated from the p-cell surface by two basal laminae, one enclosing the nerves and the other enclosing the acini, and accounting for a distance of ~0.5 μm. In addition, serotonergic fibers invade the acini and form a dense network between c-cells. Notably, every c-cell seems to have (only) serotonergic synapses on its surface. Nerves between acinar lobules contain many dopaminergic and only a few serotonergic release sites and may thus serve as neurohemal organs. Salivary duct segments immediately following the acini are locally associated with dopaminergic and serotonergic fibers and release sites on their surface and between duct epithelial cells. Duct segments further downstream have only a sparse dopaminergic innervation. The reservoir sacs and the reservoir muscles also have a dopaminergic and a serotonergic innervation. This innervation pattern is consistent with the view that c-cells respond only to 5-HT, p-cells to 5-HT, and DA, and most duct cells only to DA. In addition, these observations suggest that c-cells are stimulated by 5-HT released close to their surface in synapse-like structures, whereas p-cells and most duct cells are exposed to 5-HT and DA released from varicosities some distance away.

Despite our recent systematic studies of the innervation of the salivary glands, a number of questions remain unanswered: (1) What is the physiological significance of the dual serotonergic innervation by nerve 7b and the stomatogastric nervous system? (2) Do serotonergic fibers from both

sources innervate the same acini? (3) What is the exact innervation pattern and the physiological role of the innervation by SN2? (4) What is the molecular identity and physiological function of co-transmitters that may be released with 5-HT and DA, as suggested by the finding that each release site on the salivary gland contains both clear and dense-core vesicles (Maxwell, 1978; Baumann et al., 2004)?

DA- AND 5-HT-INDUCED SALIVA PRODUCTION: AN OVERVIEW

The discovery that stimulation with 5-HT results in the production of a protein-rich saliva, whereas stimulation with DA causes production of protein-free saliva, was extremely helpful for understanding the physiology of cockroach salivary glands (Just and Walz, 1996). These observations emphasize that saliva production is highly complex, a feature that has to be addressed in future investigations. The integration of recent findings with older data now permits a rough description of the sequence of events and the mechanisms involved in acinar fluid secretion and the modification of the primary saliva as it flows through the ducts (summarized in Fig. 2).

Electrical stimulation of the salivary duct nerve or superfusion of isolated glands with DA or 5-HT induces saliva secretion (Just and Walz, 1996; Rietdorf et al., 2003; for a review of older literature, see House, 1980). The rate of fluid secretion is governed largely by the secretory activity of the p-cells. These cells hyperpolarize (from ca. –55 mV up to –100 mV; House, 1975) upon stimulation with DA, probably because of the activation of a Ca^{2+}-dependent K^+ conductance ($g_{K,Ca}$ in Fig. 2) (Ginsborg et al., 1980a,b). The primary saliva secreted into the acinar lumen of DA-stimulated glands is almost iso-osmotic with physiological saline (see Table 1; Gupta and Hall, 1983). Gupta

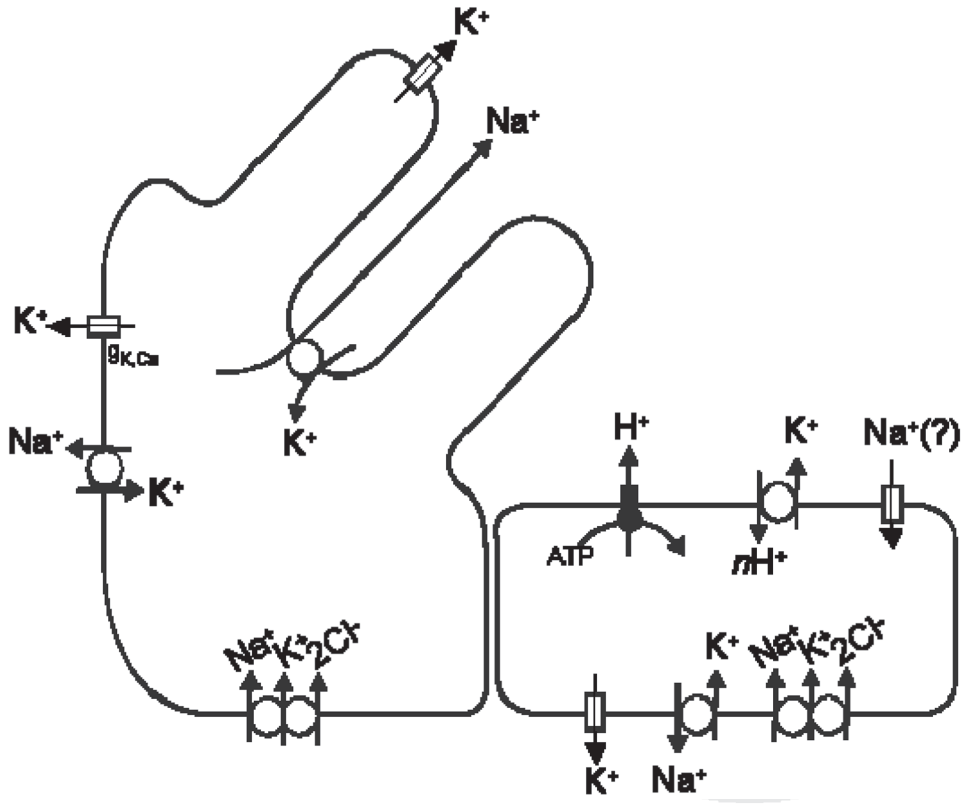

Fig. 2. Schematic drawing of a p-cell (**left**) and a duct cell (**right**). These two cell types are involved in the secretion of the primary saliva and its subsequent modification. Ion channels and transporters that have been identified electrophysiologically, pharmacologically, and/or immunocytochemically are indicated. For details, see text.

and Hall (1983) suggested that a Na^+/K^+-ATPase located in the apical microvilli of the p-cells drives the transepithelial NaCl and H_2O transport. The presence of a Na^+/K^+-ATPase at this place has been confirmed immunocytochemically (Just and Walz, 1994b). In addition, the microvillar membrane should contain a K^+ conductance for apical K^+ recycling. The observation that bumetanide, a specific blocker of the $Na^+/K^+/2Cl^-$ co-transporter, strongly reduces the rate of fluid secretion and the ionic content and osmolarity of the final saliva after DA and 5-HT stimulation strongly suggests that this co-transporter is also important for Na^+, K^+, and Cl^- entry into p-cells and fluid secretion (Rietdorf et al., 2003). In addition, bath application of ouabain increases the secretory rate of 5-HT- and DA-stimulated glands, indicating that there is also a Na^+/K^+-ATPase activity in the basal membrane as suggested by Gupta and Hall (1983). However, the basolateral Na^+/K^+-ATPase is not detectable immunocytochemically, either because its concentration is too low, or it has not been detected by the antibodies raised to date.

Compared with primary saliva and physiological saline, the final saliva is hyposmotic (see Table 1; Rietdorf et al., 2003). Obviously, the duct cells modify the primary saliva by re-absorption of ions and/or water. In addition, since the Na/K ratio in the final saliva drops to ~1/3 of the ratio in the primary saliva, the ducts reabsorb Na^+ and secrete K^+ (Gupta and Hall, 1983; Rietdorf et al., 2003). For these changes to occur, an apical vacuolar-type H^+-ATPase, a $K^+/n{>}H^+$-antiporter, a basal Na^+/K^+-ATPase, and a basal $Na^+/K^+/2Cl^-$-co-transporter have been suggested to be involved (Just and Walz, 1994b; Lang and Walz, 1999, 2001; Rietdorf et al., 2003).

TABLE 1. Ionic Composition of *Periplaneta americana* Primary and Final Saliva Upon DA Stimulation*

	Primary saliva (mM)	Final saliva (mM)
$[Na^+]$	153	95
$[K^+]$	26	38
[Cl]	172	145

*Values for primary saliva were obtained by using electron probe X-ray microanalysis of frozen-hydrated and frozen-dried cryosections and are taken from Gupta and Hall (1983). Ion concentrations for final saliva were measured by capillary electrophoresis of saliva samples and are taken from Rietdorf et al. (2003).

STIMULUS-SECRETION COUPLING

Our knowledge about the signaling cascades that couple electrolyte/water secretion, protein secretion, and the modification of the primary saliva to biogenic amine stimuli is still fragmentary. Since saliva production is mediated by various cell types that are innervated by dopaminergic and/or serotonergic fibers, the signaling mechanisms in every cell type have to be examined separately.

Acinar p- and c-cells hyperpolarize upon stimulation with DA (Bowser-Riley and House, 1976; House, 1975). The hyperpolarization and resulting fluid secretion are Ca^{2+}-dependent. Electrophysiological studies have shown that the DA-induced

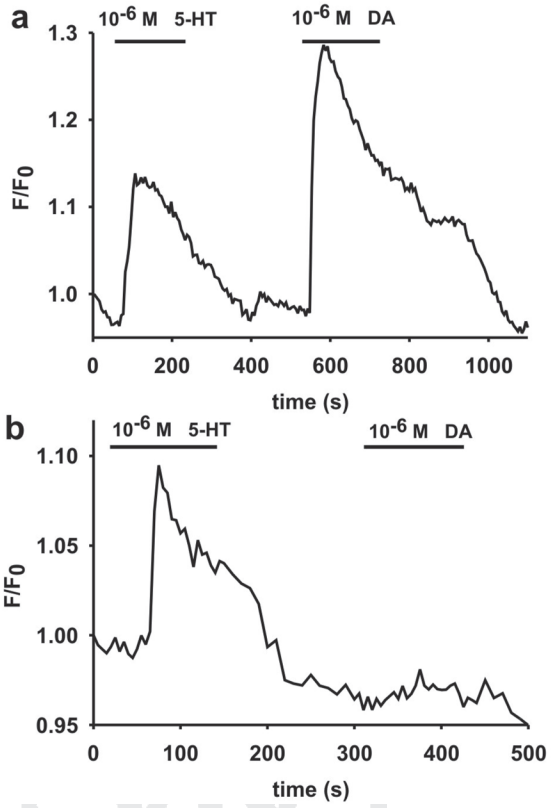

Fig. 3. Serotonin (5-HT)- and dopamine (DA)-induced intracellular calcium changes in p-cells (**a**) and c-cells (**b**). Both p- and c-cells were isolated and loaded with the Ca^{2+}-sensitive dye Rhod-2. Superfusing the cells with 1 µM 5-HT induces a Ca^{2+} elevation in both p- and c-cells. In contrast, superfusing the cells with 1 µM DA induces Ca^{2+} signals only in p-cells.

Ca^{2+} elevation is partially attributable to Ca^{2+} release from intracellular stores (Ginsborg et al., 1980b). We succeeded in recording amine-induced Ca^{2+} changes in isolated p- and c-cells that were loaded with the Ca^{2+}-sensitive dye Rhod-2 (Fig. 3). Whereas 5-HT induced a Ca^{2+} elevation in p- and c-cells, Ca^{2+} responses to DA application were observed only in p-cells. This result nicely corroborates the innervation pattern of p- and c-cells from which one assumes that DA only stimulates p-cells. The way in which the DA- and 5-HT-induced Ca^{2+} elevation is involved in the activation of transepithelial Na^+ and Cl^- transport by the p-cells is unknown. DA has also been shown to stimulate cAMP production in the salivary glands of *Nauphoeta cinerea* (Grewe and Kebabian, 1982). Unfortunately, the cell type(s) in which the cAMP concentration ($[cAMP]_i$) increases is/are unknown.

Pharmacological experiments on isolated *Periplaneta* salivary glands have demonstrated that protein secretion from c-cells requires an increase in the intracellular concentrations of both cAMP and Ca^{2+} (Rietdorf et al., in press). How the increase in the concentration of these second messengers is linked to the biochemical machinery leading to exocytosis of secretory granules remains to be determined.

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The situation is likewise complex and puzzling in the dopaminergically innervated epithelial cells of the ducts that modify the primary saliva. Stimulation of isolated glands with 1 µM DA causes the basolateral membrane potential of duct epithelial cells to depolarize from –67 ± 1 to –41 ± 2 mV. In addition, DA causes a dose-dependent increase in the intracellular Ca^{2+} concentration that spreads over the duct epithelium as a "Ca^{2+}-tide." The Ca^{2+} elevation does not occur in Ca^{2+}-free solution and is blocked by bath application of La^{3+}, an unspecific blocker of Ca^{2+} channels (Lang and Walz, 1999a). Compared with acinar cells, the electrical response and the Ca^{2+} entry site are different in duct cells. At the molecular level, the target molecules and transporters that are activated by Ca^{2+} remain to be identified. It is also unknown whether cAMP is involved in the activation of duct cells.

PHARMACOLOGY OF AMINE EFFECTS

The pharmacological properties of aminergic receptors involved in saliva secretion can be investigated by various techniques. The responses of isolated glands exposed to agonists, antagonists, and combinations of both applied to the bathing solution can be monitored. Furthermore, the effect of antagonists on electrically stimulated nerve-gland preparations can also be examined. These treatments either cause (1) changes in the rate of fluid secretion by isolated glands, (2) changes in the membrane potential, conductance, or capacity of gland cells, or (3) changes in the concentration of second messengers in the gland cells. Earlier findings on the pharmacological properties of receptors on the salivary gland of the cockroach *Nauphoeta cinerea* have been reviewed in detail by House and Ginsborg (1979, 1985) and House (1980). Briefly, DA and the non-selective DA receptor agonist 6,7-ADTN are the most potent agonists producing a half-maximal electrical and secretory response at a concentration of ~50 nM. This result in combination with the finding that *cis*(Z)-flupenthixol, a non-selective DA receptor antagonist, is the most potent blocker of glandular responses (House and Ginsborg, 1976; Breward et al., 1980) has suggested that specific DA receptors mediate the secretory and electrical events caused by nerve stimulation (House and Ginsborg, 1976; House, 1980). A structure-activity study has shown that the two catechol OH groups are necessary but not sufficient for agonist action, because *N*-acetyldopamine is inactive (Ginsborg et al., 1976b).

In mammals, DA binds to two subfamilies of DA receptors: D1- and D2-(like) receptors (Kebabian and Calne, 1979). They can be distinguished by their pharmacological properties and intracellular signaling pathways. D1 and D5 receptors constitute the D1-subfamily and activate adenylate cyclase, whereas members of the D2-subfamily, i.e., the D2, D3, and D4 receptors, either inhibit adenylate cyclase or couple to different intracellular second messenger systems (for reviews, see: Seeman and Van Tol, 1994; Missale et al., 1998; Callier et al., 2003). In the early 1990s, the pharmacologi-

cal properties of DA receptors in the salivary glands of *Nauphoeta cinerea* were investigated by Evans and Green (1990a,b, 1991) and compared with mammalian DA receptors. The authors described the following rank order of agonists: DA >> fenoldopam (D1-like) > SKF 38393 (D1-like) > quinpirole (D2-like) (Evans and Green, 1991). The rank order of antagonists was: chlorpromazine (non-selective) > SCH 23390 (D1-like) ≥ haloperidol (D2-like) > metoclopramide (D2-like) (Evans and Green, 1990a,b). Domperidone (D2-like) and (±)-sulpiride (D2-like) were inactive (Evans and Green, 1990a,b). From these data, Evans and Green concluded that only one class of receptor with pharmacological properties similar to those of mammalian D1 receptors mediate the effects of DA in *Nauphoeta* salivary glands. This is in good agreement with the findings that DA increases $[cAMP]_i$ in the salivary glands of *Nauphoeta* (Grewe and Kebabian, 1982) and that cAMP in the bathing solution causes a dose-dependent secretory response (Gray et al., 1984). Interestingly, cAMP fails to hyperpolarize the acinar cell membrane potential as DA does (Gray et al., 1984). An explanation could be that the D1-like receptors in the *Nauphoeta* salivary gland are coupled to phospholipase C (PLC) in addition to adenylyl cyclase (Evans et al., 1991). IP_3 synthesized by PLC seems to be involved in mediating both the secretory and electrical response to DA (Evans et al., 1991). D1-like dopamine receptors have been identified in the salivary glands of locusts (Keating and Orchard, 2001, 2004) and ticks (Schmidt et al., 1981, 1982; for reviews, see Sauer et al., 2000; Bowman and Sauer, 2004) as well.

House and co-workers recognized that 5-HT also induces secretion in the salivary glands of *Nauphoeta* and provided the first evidence that 5-HT "interacts with different receptors from those for the other agonists" (House et al., 1973; Bowser-Riley et al., 1978; House, 1980). Interestingly, the electrical but not the secretory response could be blocked by the ergoline derivative ergometrine (Ginsborg et al., 1976a; Bowser-Riley et al., 1978), which is not only a DA-receptor antagonist (Ascher, 1972), but also a potent agonist at certain mammalian 5-HT-receptor subtypes (Brazenor and Angus, 1982; Bai et al., 2004). These pharmacological results support the hypothesis that at least two classes of aminergic receptors are expressed on the salivary gland of *Nauphoeta* (House, 1980). In *Periplaneta*, DA and 5-HT are known to control the secretion of protein-free and protein-containing saliva, respectively (Just and Walz, 1996). By measuring both the secretory rate and protein content

TABLE 2. Pharmacological Properties of Salivary Gland DA-Receptors in *Periplaneta**

DA receptor ligand	Specificity in vertebrates	Secretory rate in relation to a preceding control stimulation with 1 μM DA (= 100%) (%)
Agonists		
DA (1 μM)		79.9 ± 6.3
6,7-ADTN (1 μM)	DA receptor agonist	90.1 ± 7.6
R(+)-Lisuride (1 μM)	Non-selective DA receptor agonist; agonist at 5-HT_{1A} and 5-HT_{1B} receptors and antagonist at 5-HT_7 receptors	32.3 ± 3.1
Chloro-APB (1 μM)	Full D_1 DA receptor agonist	24.3 ± 6.8
Chloro-APB (10 μM)		69.4 ± 3.6
R(+)-SKF 38393 (1 μM)	D_1 DA receptor agonistq	3.1 ± 1.3
R(+)-SKF 38393 (10 μM)		3.6 ± 1.3
R(–)-TNPA (1 μM)	Potent, selective D_2 DA receptor agonist	0.3 ± 0.2
R(–)-TNPA (10 μM)		12.1 ± 2.2
Agonists (in combination with 1 μM DA)		
None		79.9 ± 6.3
cis(Z)-Flupenthixol (1 μM)	DA receptor antagonist	56.0 ± 3.8
cis(Z)-Flupenthixol (10 μM)		17.4 ± 8.2
Chlorpromazine (1 μM)	D_2 DA receptor antagonist	61.0 ± 2.9
Chlorpromazine (10 μM)		28.2 ± 17.7
S(+)-Butaclamol (1 μM)	DA receptor antagonist	53.7 ± 11.2
S(+)-Butaclamol (10 μM)		30.6 ± 12.1

*The effect of various DA-receptor agonists and antagonists on saliva secretion of isolated *Periplaneta* salivary glands was measured. Values are from Marg et al. (2004). Note the low efficacy of subtype-specific DA-receptor ligands.

of the saliva, Marg et al. (2004) have been able to show whether ligands specifically act on p-cells or simultaneously on p- and c-cells (see Table 2). The effects of DA can be mimicked by the non-selective DA receptor agonist 6,7-ADTN and, less effectively, by the D1-like agonist chloro-ABP. The D1-like agonist SKF 38393 and D2-like agonist R(−)-TNPA are ineffective. R(+)-lisuride induces a secretory response with a slower onset and a lower maximal response compared with DA-induced secretion. Saliva secreted after stimulation with ADTN, lisuride, or chloro-ABP has no measurable protein content, arguing against 5-HT receptors being activated by these drugs. DA-induced secretion can be blocked by DA-receptor antagonists *cis*(Z)-flupenthixol, chlorpromazine, and S(+)-butaclamol. This pharmacological profile is remarkably different from that of vertebrate DA receptors and does not unequivocally distinguish whether the receptors on *Periplaneta* salivary glands are D1- or D2-like receptors. Unfortunately, nothing is known about the pharmacological properties of 5-HT receptors associated with the salivary glands of either *Periplaneta* or *Nauphoeta*.

MOLECULAR CHARACTERIZATION OF AMINE RECEPTORS

Recently, we have started to identify biogenic-amine receptors of *Periplaneta* by molecular methods. Our goal is to characterize the pharmacological profiles, the second-messenger coupling, and the spatial expression patterns of identified receptors. Most biogenic amine receptors belong to the su-

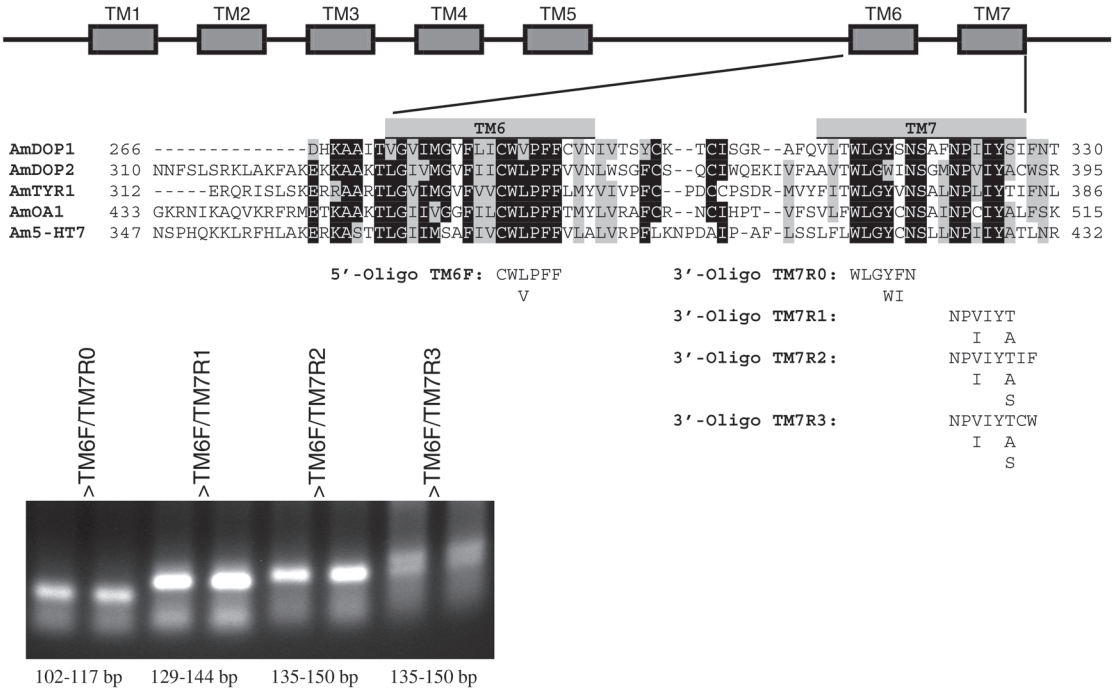

Fig. 4. Molecular analysis of biogenic-amine receptors of *Periplaneta*. A biogenic amine receptor typically spans the cell membrane seven times. These transmembrane segments (TM1–7) are depicted as cylinders. Amino-acid sequence alignment of five biogenic-amine receptors cloned from honeybee (*Apis mellifera*, Am) brain. For clarity, shown is only the alignment from TM6 to TM7: AmDOP1 = DA receptor 1 (Blenau et al., 1998), AmDOP2 = DA receptor 2 (Humphries et al., 2003), AmTYR1 = tyramine receptor 1 (Blenau et al., 2000), AmOA1 = octopamine receptor 1 (Grohmann et al., 2003), Am5-HT7 = serotonin 5-HT7 receptor (J. Schlenstedt et al., unpublished data). Based on the conserved amino-acid motives depicted below the alignment, degenerate oligonucleotides were synthesized for PCR amplification of receptor fragments. **Bottom:** PCR amplification products with a *Periplaneta*-brain cDNA library as template.

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perfamily of G protein–coupled receptors (Blenau and Baumann, 2001). These receptors share the common motif of seven transmembrane (TM) domains. Specific amino-acid residues located in TM segments participate in ligand binding. The TM segments are highly conserved between orthologous receptors of various species. Multiple alignments of amino acid sequences of ~30 different insect biogenic-amine receptors have been performed to design degenerate oligonucleotide primers that correspond to highly conserved amino-acid sequences within TM6 and TM7. Figure 4 depicts the alignment of five sequences from *Apis mellifera*. Polymerase chain reaction (PCR) amplification from oligo dT-primed *Periplaneta* brain cDNA resulted in products of the expected length (see Fig. 4). The PCR products usually represent mixtures of various biogenic-amine receptor fragments. Indeed, sequence analysis has shown that four different biogenic-amine receptors were cloned (Fig. 5): one DA receptor (PeaDOP2), one 5-HT receptor (Pea5-HT1), one tyramine receptor (PeaTYR), and one fragment (Pea4a) corresponding to a non-characterized *Drosophila* receptor (CG31350).

Although the full-length cDNAs have to be cloned and functionally characterized after heterologous expression, the identification of DA- and 5-HT receptor fragments will allow us to examine whether and where exactly these genes are expressed in the salivary glands of *Periplaneta*. We expect that the combined application of molecular, pharmacological, and physiological approaches will enable us to unravel precisely the signaling cascades triggered by biogenic amines in p- and c-cells of the salivary glands within the foreseeable future.

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Protein secretion in cockroach salivary glands requires an increase in intracellular cAMP and Ca^{2+} concentrations

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Abstract

The salivary glands in the cockroach *Periplaneta americana* secrete protein-containing saliva when stimulated by serotonin (5-HT) and protein-free saliva upon dopamine stimulation. In order to obtain information concerning the signalling pathways involved in 5-HT-induced protein secretion, we have determined the protein content of saliva secreted after experimental manipulations that potentially elevate intracellular Ca^{2+} and cyclic nucleotide concentrations in isolated glands. We have found that 5-HT stimulates the rate of protein secretion in a dose-dependent manner (threshold: 3×10^{-8} M; EC_{50} 1.5×10^{-6} M). The maximal rate of 5-HT-induced protein secretion was 2.2 ± 0.2 µg/min. Increasing intracellular Ca^{2+} or cAMP by bath application of ionomycin (5 µM), db cAMP (10 mM), forskolin (100 µM) or IBMX (100 µM), respectively, stimulated protein secretion at significantly lower rates, whereas db cGMP (1 mM) did not activate protein secretion. The high rates and the kinetics of 5-HT-induced protein secretion could only be mimicked by either applying forskolin together with IBMX (with or without ionomycin) or by applying IBMX together with ionomycin. Our measurements suggest that 5-HT-induced protein secretion is mediated by an elevation of $[cAMP]_i$ and that Ca^{2+} may function as a co-agonist and augment the rate of protein secretion.

Keywords: Salivary gland; Insect; Protein secretion; Serotonin; Calcium; cAMP

1. Introduction

Although insect salivary glands were studied extensively in the 1970s and 1980s (for reviews, see House, 1980; House and Ginsborg, 1985), the whole range of mechanisms from their neuronal or hormonal stimulation to saliva secretion is not yet understood. In salivary glands, the term stimulus-secretion-coupling encompasses the signalling pathways that mediate and induce two fundamentally different processes involved in saliva production: (1) electrolyte and water secretion, and (2) protein secretion.

In many exocrine glands, such as the salivary glands, the processes of electrolyte and water secretion occur in the same cells as protein secretion. Pancreatic acinar cells, for example, produce the primary pancreatic fluid and secrete digestive proteins into it (Thévenod, 2002). Similarly, in mammalian salivary glands, primary saliva production and the exocytosis of digestive enzymes is often carried out by the same cell type, although possibly in slightly different regions of the gland (for a review, see, Gallacher and Petersen, 1983). In such systems, it is often difficult to disentangle the mechanisms that are responsible for the regulation of either electrolyte/water transport or protein secretion. In the cockroach salivary glands, in contrast, fluid and protein secretion are carried out by different cell types and can be selectively stimulated by different neurotransmitters. Dopaminergic and serotonergic fibres innervate the gland (Baumann et al., 2002, 2004; Elia et al., 1994). The stimulation of isolated glands with dopamine induces the production of protein-free saliva, which is

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secreted exclusively by the acinar peripheral cells (p-cells) (Just and Walz, 1996; Marg et al., 2004). On the other hand, serotonin (5-hydroxytryptamine, 5-HT) stimulates acinar p-cells and central cells (c-cells) (Bowser-Riley et al., 1978; House and Smith, 1978; Bowser-Riley and House, 1976; Just and Walz, 1996). The latter secrete proteins into the acinar lumen leading to the production of protein-containing saliva (Just and Walz, 1996).

The mechanisms of electrolyte and water secretion in cockroach salivary glands are fairly well understood (Gupta and Hall, 1983; Lang and Walz, 1999, 2001; Rietdorf et al., 2003; for reviews, see, House, 1980; Walz et al., in press) but little is known about the mechanisms that mediate between the serotonergic stimulation and the secretion of salivary proteins in the salivary glands of cockroaches and other insects. We have therefore used the cockroach salivary glands as a model for obtaining information about the signalling pathways involved in protein secretion by c-cells. In particular, we are interested in the possible involvement of the second messengers Ca^{2+} and/or cyclic nucleotides. For this purpose, we have determined the protein content in the saliva secreted from isolated glands by a biochemical assay after experimental manipulations that potentially elevate intracellular Ca^{2+} ($[Ca^{2+}]_i$) and cyclic nucleotide concentrations.

2. Materials and methods

2.1. Animals and tissue preparation

A colony of *Periplaneta americana* (L.) (Blattodea, Blattidae) was reared at 27 °C under a light:dark cycle of 12:12 h. The animals had free access to food and water. Only male imagines aged between 1 and 12 weeks were used for the experiments.

Dissection of the glands was performed in physiological saline (PS) as described elsewhere (Just and Walz, 1994; Rietdorf et al., 2003; Marg et al., 2004). Isolated glands with their reservoirs removed were transferred to a PS-filled Perspex chamber (volume ~0.5 ml) and continuously superfused with oxygenated PS at a rate of about 0.5 ml/min. The main salivary duct was carefully drawn across a dividing barrier and pinned to the base of a second compartment, which was filled with liquid paraffin. For each experiment, three glands were dissected and, one after another, transferred into one Perspex chamber and stimulated simultaneously. Saliva droplets were collected from the cut end of the salivary duct by using gel loader tips every 2.5 min and transferred to a second pool of liquid paraffin.

2.2. Measurements of fluid secretion

For the measurement of the rates of fluid secretion, the longitudinal and transverse diameters (a and b, respectively) of the often ellipsoid saliva droplets were measured by using a CCD video camera (Hamamatsu, Herrsching, Germany) attached to a LEICA MZAPO stereo microscope and an Argus-10 image processor (Hamamatsu, Herrsching, Germany). Secretory rates (nl/min) were calculated from the volume (V) of the ellipsoid saliva droplets, which was calculated according to the equation $V = 4/3\,\pi ab^2$.

After measurements of the diameters, saliva samples were transferred into an Eppendorf tube filled with paraffin oil and stored at −20 °C until the protein assay was performed.

2.3. Protein assay

For measuring the protein content of saliva samples, a modified Bradford assay (Bradford, 1976) was performed. Roti Nanoquant solution (Roth, Karlsruhe, Germany) was used as the reagent according to the suppliers' instructions. Each droplet of saliva was transferred to one well of a 96-well microtiter plate and diluted with 50 µl H_2O. Saliva droplets with a volume larger than 1 µl, which seemed to have high protein content (according to their viscosity), were first diluted in H_2O and thereafter 50 µl of the diluted sample was transferred into the wells. Roti Nanoquant (200 µl) working solution was added to each well. After 5 min, absorption at 450 and 650 nm was measured in a Milenia Kinetic Analyzer (Diagnostic Products Corporation, Los Angeles, CA, USA). For complete mixture of the samples and solutions, plates were shaken in the analyzer three times prior to measurement.

Each microtiter plate was calibrated. The following concentrations of bovine serum albumin (BSA) were used as standards for the calibration: 0, 20, 30, 40, 50, 60, 80 and 100 µg BSA/ml. Aliquots (50 µl) of each standard were transferred into the wells of a microtiter plate. Because there were always small amounts of paraffin oil transferred together with the saliva droplets, about 3 µl paraffin oil was added to each well containing a standard. This corresponded to the maximum amount of transferred paraffin oil. Absorption of the standards was measured together with the samples. The ratio of absorption at 650/450 nm was used for analysis. A linear regression curve was calculated for the standard solutions and used for quantification of protein content in the samples when R^2 was >0.99. The lower detection limit was 10 µg/ml, corresponding to 500 ng protein in a saliva sample; the upper detection limit was 100 µg/ml, which corresponded to 5 µg protein in a saliva sample.

2.4. Chemicals and solutions

Standard cockroach PS contained (in mM): 160 NaCl, 10 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 glucose, and 10 Tris; the pH was adjusted to 7.4 by HCl.

For stimulation of the glands, 1 μM dopamine or 5-HT was used. Dopamine, 5-HT and MDL 12,330 A were dissolved in H_2O. Stock solutions (10 mM) were prepared in ethanol for 3-isobutyl-1-methylxanthine (IBMX) and forskolin and in DMSO for ionomycin. Dibutyryl cAMP (db cAMP) and cAMP were dissolved at their final concentration in PS (adjusted to pH 7.4). All stock solutions were stored at −20 °C in small aliquots and diluted to their final concentrations in PS immediately prior to use. All chemicals were of analytical grade.

2.5. Data analysis and statistics

For the calculation of the dose-response curve, software XACT (Version 7.22a, SciLab) was used. A sigmoidal curve was fitted through the values (by using error minimizing according to the least square protocol). Statistical analysis was performed by using the software Statistica (Version 4.5, Statsoft Inc., 1995). Data are given as mean ± SE; $p \leqslant 0.05$ was used as the level for statistical significance.

Data from individual glands were not used for the analysis when one of the following criteria applied: (1) there were still proteins in the saliva, 5 min after the onset of the dopamine stimulation, (2) the rate of fluid secretion during the first 10 min of dopamine stimulation did not raise above 100 nl/min or (3) the rate of fluid secretion dropped below 100 nl/min more than once during the stimulation of the glands.

3. Results

All experiments required a continuous flow of saliva in order to wash-out protein secreted by c-cells effectively and continuously, in particular under experimental conditions that may stimulate protein secretion but little fluid secretion. For example, during stimulation with low doses of 5-HT (Just and Walz, 1996) or other substances that stimulate fluid secretion at very low rates it is difficult to collect enough saliva for reliable determinations of its protein content. Therefore, in this study, continuous saliva production was achieved by background stimulation with 1 μM dopamine.

3.1. Kinetics of 5-HT-induced protein secretion and dose-response relationship

When 1 μM 5-HT was applied on a background of 1 μM dopamine, it caused a transient increase in the rate of protein secretion (Fig. 1). The maximal rate of 2.2 ± 0.2 μg/min (Table 1) was reached 18 ± 2 min after the beginning of stimulation with 5-HT. A dose-response relationship for 5-HT was determined by using the following stimulation protocol. Every gland was stimulated with 5-HT twice in the continuous presence of 1 μM dopamine. An initial stimulation with 1 μM 5-HT during 10–20 min of the experiment served as a control and reference, respectively. Afterwards, between 20 and 45 min, 5-HT was washed out, and protein secretion declined completely. Variable 5-HT concentrations were applied during a second stimulation period between 45 and 75 min. Maximal rates of protein secretion during the second 5-HT stimulation period were normalized to the maximal rate measured in the same gland during the first stimulation period (100%). These normalized values were taken for the dose-response relationship illustrated in Fig. 2A. The dose-response relationship shows that a second application of 5-HT is less effective in stimulating protein secretion than the first (the second application of 1 μM 5-HT is only ~70% as effective as the first application of 1 μM 5-HT). Therefore, and to avoid normalizing protein secretion rates elicited by the test 5-HT concentration to a reference with different stimulation history, secretory rates were normalized to the secretion rates produced by the first 5-HT application. Fig. 2A shows that the threshold concentration of 5-HT that activated measurable protein secretion was 3×10^{-8} M under these conditions. The protein secretion rate was half-maximal at 1.5×10^{-6} M 5-HT and saturated at 5-HT concentrations of $\geqslant 10^{-4}$ M.

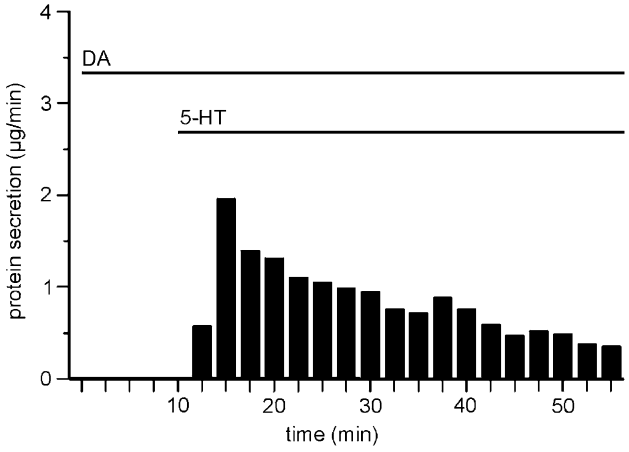


Fig. 1. Protein secretion activated by 1 μM 5-HT (data from one representative experiment). Background stimulation with 1 μM dopamine was applied throughout the experiment. Exactly 10 min after starting to stimulate the glands with dopamine, 1 μM 5-HT was added. Protein secretion could be detected at the first measurement point after the addition of 5-HT (at 12.5 min on the graph) and reached a maximum 5 min after addition of 5-HT. Values are given in μg/min.

Table 1
Protein secretion following treatment with 5-HT or an increase in putative second messengers $[Ca^{2+}]_i$, $[cAMP]_i$ or $[cGMP]_i$

Treatment	Protein secretion (μg/min)	*P*	*n*
5-HT	2.2±0.2		12
To increase $[Ca^{2+}]_i$			
Ionomycin	0.4±0.1	*	6
To increase $[cAMP]_i$			
db cAMP	0.2±0.002	n.t.	3
Forskolin	0.4±0.04	*	9
IBMX	0.7±0.1	*	8
Forskolin & IBMX	1.4±0.2	n.s.	9
To increase $[cGMP]_i$			
db cGMP	0.0	n.t.	4
db cGMP & ionomycin	0.0	n.t.	4
db cGMP & IBMX	0.8±0.1	*	9
To simultaneously increase $[Ca^{2+}]_i$ and $[cAMP]_i$			
Forskolin & ionomycin	0.9±0.1	*	8
IBMX & ionomycin	1.7±0.4	n.s.	7
Forskolin, IBMX & ionomycin	1.6±0.3	n.s.	7

Values are given as mean±SE (μg/min). The following concentrations of drugs were applied: 5-HT, 1 μM; ionomycin, 5 μM; IBMX, 100 μM; forskolin, 100 μM; db cGMP, 1 mM; db cAMP, 10 mM. Continuous background stimulation with 1 μM dopamine was applied in all experiments. Secretory rates upon various treatments were statistically compared with the maximal protein secretion rate upon stimulation with 1 μM 5-HT (one-way analysis of variance; n.s. $P>0.05$; *$P\leqslant 0.05$; n.t. not tested because of the low number of experiments; n = number of glands tested.

The kinetics in the rate of protein secretion also varied with the 5-HT concentration (Fig. 2B). Glands stimulated with low (e.g. 7×10^{-8} M) 5-HT concentrations displayed protein secretion at a low steady rate. In contrast, higher 5-HT concentrations ($\geqslant10^{-6}$ M) produced a transient increase in the rate of protein secretion, whereby the time-to-peak decreased with increasing 5-HT concentrations.

3.2. Does an elevation of intracellular Ca^{2+} or cyclic nucleotide concentration stimulate protein secretion?

Substances that potentially elevate intracellular Ca^{2+} or/and cAMP concentrations were applied for 45 min (10–55 min) in the continuous presence of 1 μM dopamine (see Section 3.1) as illustrated in Fig. 3. At the end of every experiment, 1 μM 5-HT was applied only in order to test whether the gland was still capable of secreting protein after the prolonged stimulation with the test substance. For purposes of comparison, however, mean rates of protein secretion presented in Table 1 were normalized to the mean rate elicited by 1 μM 5-HT in separate control experiments, where 5-HT was applied on the dopamine background at 10 min (and not at the end of the experiment).

Bath application of the Ca^{2+} ionophore ionomycin (5 μM) induced protein secretion, but only after a delay of about 10 min. The rate of protein secretion increased very slowly (Fig. 3A) and did not exceed 0.4 ± 0.1 μg/min. This corresponded to only 18% of the secretion rate obtained by stimulation of the gland with 1 μM 5-HT (Fig. 3A and Table 1).

All pharmacological treatments that potentially elevated intracellular cAMP concentration ($[cAMP]_i$) induced protein secretion, but with notable quantitative and qualitative differences (Fig. 3 and Table 1). The membrane-permeant analogue of cAMP, db cAMP (10 mM), caused protein secretion at a low rate. It was often not even possible to measure protein in samples collected during the extended stimulation period (Fig. 3B). Application of the adenylyl cyclase activator forskolin (100 μM) or the phosphodiesterase (PDE) inhibitor IBMX (100 μM) stimulated protein secretion (Fig. 3C and D, respectively) at higher rates than that by db cAMP. For both treatments, the rates were constant over the entire stimulation period. Nevertheless, secretion rates were significantly lower than after stimulation with 1 μM 5-HT (Table 1). However, when forskolin and IBMX were applied together, the maximal rate of protein secretion and its transient kinetics were not significantly different from 5-HT-stimulated protein secretion (Fig. 3E and Table 1).

Thus, an elevation of $[Ca^{2+}]_i$ with ionomycin, the bath application of db cAMP alone, the activation of adenylyl cyclase with forskolin alone or the inhibition of PDE with IBMX alone were all unable to stimulate protein secretion at rates that mimicked those induced by stimulation with 5-HT. Therefore, we tested whether a simultaneous elevation of $[Ca^{2+}]_i$ and $[cAMP]_i$ produced rates of protein secretion resembling that after 5-HT treatment. The rates of protein secretion

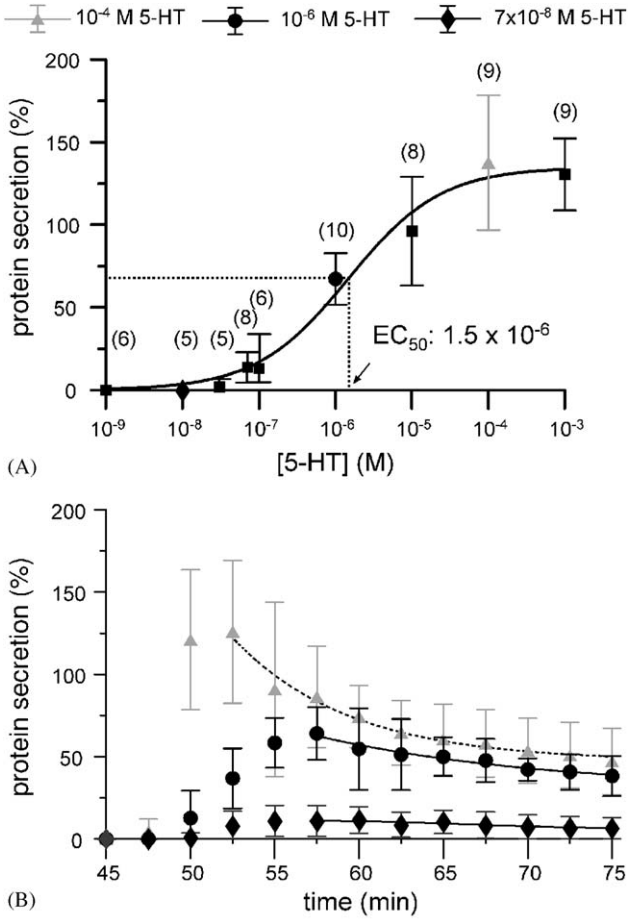


Fig. 2. (A) Dose-response curve of 5-HT-stimulated protein secretion. Protein secretion rates during the second stimulation period with 5-HT were normalized to those of the same glands during the first stimulation with 5-HT and are given as means ± SE. Values in brackets give the sample size for each 5-HT-concentration. Continuous background stimulation with 1 μM dopamine was applied in all experiments. (B) Kinetics of protein secretion after stimulation with 7×10^{-8} M (grey diamonds in A and B), 10^{-6} M (black circles in A and B) and 10^{-4} M (grey triangles in A and B) 5-HT. Time scale starts upon the second addition of 5-HT and the values are normalized as described above. Values are given as means ± SE. A non-linear regression according to the Boltzmann equation was fitted through the values from the maximum of protein secretion. The addition of 7×10^{-8} M 5-HT activates a slow rise in protein secretion and the amount of secreted proteins remains almost constant over the 30-min stimulation period. Protein secretion shows a transient pattern after stimulation with the higher 5-HT-concentrations, the transient characteristics being more prominent after stimulation with the saturating concentration of 10^{-4} M 5-HT.

induced by the simultaneous application of ionomycin and forskolin showed that the effects were additive (Fig. 3F, Table 1). However, the resultant protein secretion was still significantly lower than that after 5-HT stimulation, and its time course was not as transient (Fig. 3F). IBMX applied together with ionomycin produced a transient increase in the rate of protein secretion that was not significantly different from that produced by 5-HT (Fig. 3G and Table 1). The rates of protein secretion produced by a mixture of IBMX and forskolin could not be further enhanced by the simultaneous application of ionomycin (Fig. 3 H and Table 1).

Taken together, these data suggested that 5-HT-induced cAMP formation is important for the induction of protein secretion. For a further experimental test, we applied a putative adenylyl cyclase inhibitor (MDL 12,330 A) together with 5-HT. For these experiments, glands were first stimulated with dopamine for 10 min. From 10 to 20 min, 5-HT was added and then washed out in the continuous presence of dopamine for 25 min until protein secretion stopped. Over the next 10 min, MDL 12,330 A (100 μM) was added to the dopamine background, followed by 30 min stimulation with a mixture of dopamine, MDL 12,330A and 5-HT. Rates of fluid and protein secretion were measured during the entire experiment. We found that dopamine-induced fluid secretion was inhibited by the putative adenylyl cyclase inhibitor MDL 12,330 A but, surprisingly, this substance significantly increased the rates of protein secretion to 148 ± 15% ($n = 7$) compared with the value in the absence of the inhibitor (data not shown).

Dibutyryl cGMP (db cGMP, 1 mM) applied alone or together with ionomycin did not stimulate protein secretion (Table 1). Our measurements suggested that db cGMP suppressed the low secretion rates induced by the application of ionomycin alone. Secretion rates measured after the simultaneous application of db cGMP and IBMX were not significantly different from those obtained by stimulation with IBMX alone (Table 1). These data argued against cGMP being directly involved in stimulus-secretion-coupling.

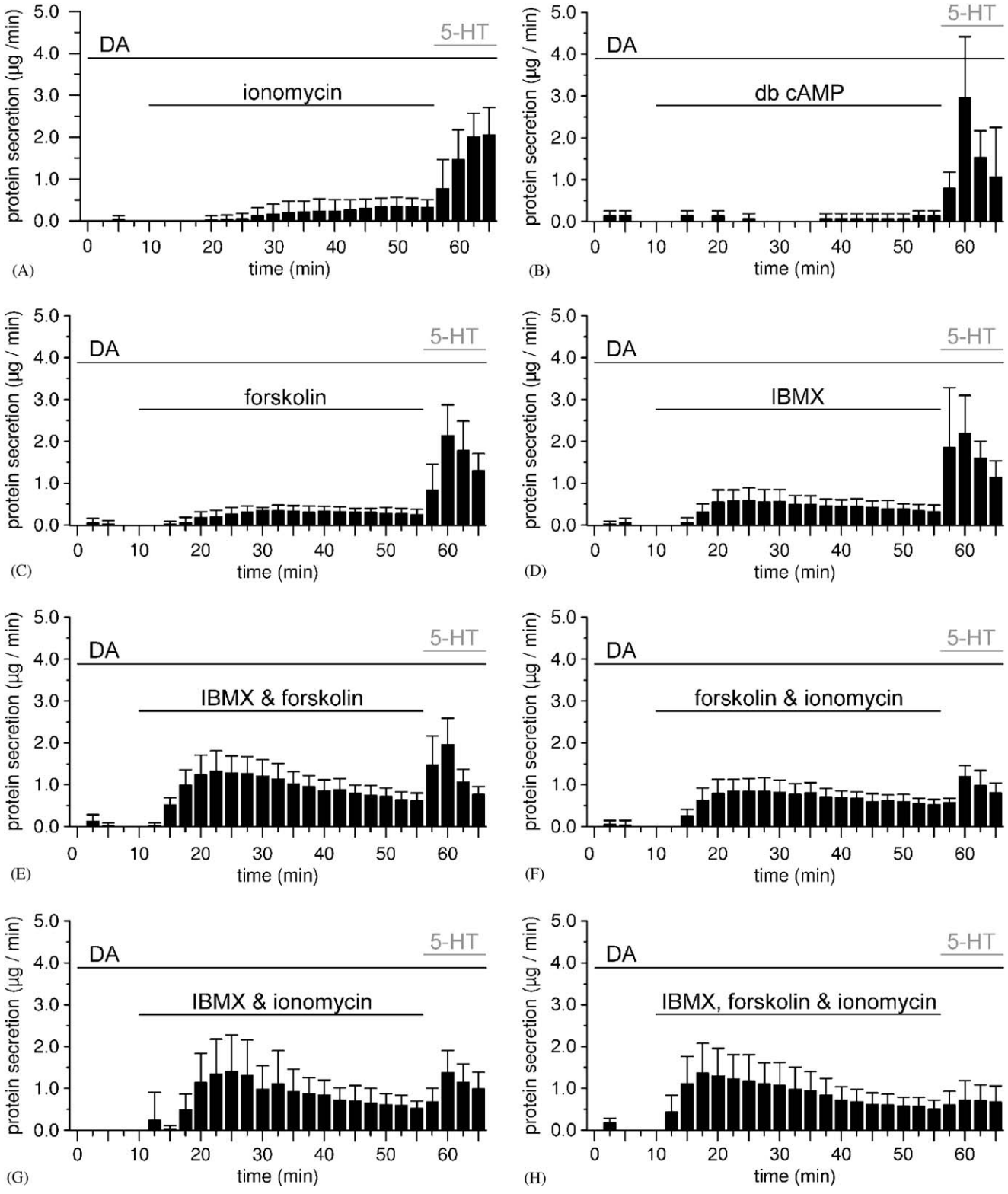
4. Discussion

We know little of the signalling pathways that lead from the stimulation of insect exocrine glands by neurohormones or neurotransmitters to exocytosis. To our knowledge, in insects the second messengers that may be involved in the regulation of protein secretion have only been characterized in the tubular salivary glands of the blowfly, *Calliphora erythrocephala* (Hansen Bay, 1978). In this gland, both fluid and enzyme secretion are stimulated by the neurohormone 5-HT. Surprisingly, the rate of enzyme secretion seemed to be independent of the 5-HT concentration (Hansen Bay, 1978).

In the present study, we have found that isolated cockroach salivary glands secrete a protein-containing saliva upon stimulation with the neurotransmitter 5-HT in a dose-dependent manner. The threshold and half-maximal concentrations of 5-HT (3×10^{-8} and 1.5×10^{-6} M, respectively) are almost identical to the

corresponding effective concentrations of 5-HT for fluid secretion (Just and Walz, 1996). This correspondence presumably ensures that the proteins that are secreted into the acinus lumen upon 5-HT stimulation are effectively transported towards and through the salivary ducts.

Hansen Bay (1978) had shown that enzyme secretion in *Calliphora* salivary glands is regulated by Ca^{2+} and



cAMP, the two second messengers that are formed upon 5-HT stimulation and that are known to induce fluid secretion (e.g. Berridge, 1977). In the morphologically more complex salivary glands of ixodid ticks the exocytosis of anticoagulant protein appears to be regulated by a prostaglandin E_2-induced increase in $[Ca^{2+}]_i$ concentration, mediated by the phosphoinositide signalling cascade and inositol 1,4,5-tris-phosphate-induced Ca^{2+} release from the endoplasmic reticulum (for a review, see, Sauer et al., 2000).

Recently, we succeeded in measuring dopamine- and 5-HT-induced Ca^{2+} changes in rhod-2 loaded p- and c-cells isolated from *P. americana* salivary glands. We found that 1 μM 5-HT induced a Ca^{2+} elevation in both, p- and c-cells. In contrast, 1 μM dopamine induced Ca^{2+} signals only in fluid-secreting p-cells (Walz et al., in press). An elevation in intracellular cAMP concentration seems to be necessary for fluid secretion in cockroach salivary glands. The neurotransmitter dopamine stimulates production of cAMP in the salivary glands of *Nauphoeta cinerea* (Grewe and Kebabian, 1982). Unfortunatelly, it is unknown, in which cell type (p- cells, c-cells, and/or duct cells) cAMP rose. However, Gray et al. (1984) have shown that exogenously applied cAMP, forskolin and IBMX produced fluid secretion in *N. cinerea* salivary glands. In fact, these authors provided evidence that in the normal course of stimulus-secretion coupling in this gland cAMP is necessary for fluid secretion, and that an increase in $[Ca^{2+}]_i$ alone is not sufficient to bring about fluid secretion.

Our experiments suggest that 5-HT-induced protein secretion is also mediated by an elevation of $[cAMP]_i$, and that Ca^{2+} may function as a co-agonist and augment the rate of protein secretion.

Experimental elevations of $[Ca^{2+}]_i$ with ionomycin in our salivary gland model do not support the notion that an elevation of intracellular Ca^{2+} is sufficient to induce protein secretion at rates that mimic 5-HT-incuced protein secretion. Although 5 μM ionomycin not only elevates $[Ca^{2+}]_i$ effectively (Zimmermann and Walz, 1997) but also stimulates fluid secretion from cockroach salivary glands (Gray et al., 1984), we observed a time lag of 10 min between the bath application of ionomycin and the start of protein secretion in *P. americana* salivary glands. In addition, the rates of protein secretion were low compared with those activated by 5-HT. We were unable to measure protein secretion in the absence of extracellular Ca^{2+}. Our experimental design is based on continuous fluid secretion maintained by background stimulation with dopamine. Gray and House (1982) have shown previously that fluid secretion by cockroach salivary glands stops immediately after removal of extracellular Ca^{2+}. Nevertheless, our results argue against Ca^{2+} being the major second messenger for protein secretion.

Alternatively, the 5-HT-induced protein secretion might be mediated by cAMP as in vertebrate salivary glands (e.g. Fujita-Yoshigaki, 1998; Hille et al., 1999; Quissell and Tabak, 1989). 5-HT increases $[cAMP]_i$ in *Calliphora* and *Locusta* salivary glands in a dose-dependent manner (Ali and Orchard, 1994; Ali et al., 1993; Prince et al., 1972). The latter show similarities in their innervation by serotonergic and dopaminergic fibres to *P. americana* salivary glands (Ali, 1997). However, the acinar locust salivary glands consist of several different cell types (Berridge and Oschman, 1972) and the cell type in which cAMP rises in response to 5-HT stimulation is unfortunately unknown.

We have used various experimental strategies to increase $[cAMP]_i$ in the c-cells of isolated *P. americana* salivary glands and have taken the rate of protein secretion as an indicator for the increase in $[cAMP]_i$. These strategies are the direct application of db cAMP, the activation of adenylyl cyclase with forskolin, the inhibition of PDE with IBMX, and a combination of these treatments. We have found that the maximal rates of protein secretion activated by these substances differ substantially; we assume that this is attributable to the different efficiencies of the substances in increasing $[cAMP]_i$.

The rate of protein secretion was maximal after the simultaneous application of IBMX and forskolin. In addition to the absolute rate, the transient pattern was also similar to that of protein secretion induced by 5-HT. This result indicates that an effective increase in $[cAMP]_i$ is necessary and sufficient to activate protein secretion in the cockroach salivary gland.

The stimulation of adenylyl cyclase with forskolin alone or the inhibition of PDE with IBMX alone

Fig. 3. Protein secretion after an induced increase in $[Ca^{2+}]_i$, $[cAMP]_i$, or both $[Ca^{2+}]_i$ and $[cAMP]_i$, respectively. Values are given as means ± SE in μg/min. The application of every test substance was followed by an application of 1 μM 5-HT at the end of each experiment to monitor the glands capability to secrete protein after a prolonged treatment with a test substance. (A) Protein secretion stimulated by an increase of $[Ca^{2+}]_i$ after addition of 5 μM ionomycin ($n = 6$). (B-E) Protein secretion stimulated by an increase of $[cAMP]_i$ after application of: (B) 10 mM db cAMP ($n = 3$), (C) 100 μM forskolin ($n = 9$), (D) 100 μM IBMX ($n = 8$), and (E) 100 μM forskolin plus 100 μM IBMX ($n = 9$). (F-H) Protein secretion activated by the simultaneous increase of $[Ca^{2+}]_i$ and $[cAMP]_i$ after application of: (F) 100 μM forskolin plus 5 μM ionomycin ($n = 8$), (G) 100 μM IBMX plus 5 μM ionomycin ($n = 7$), and (H) 100 μM forskolin plus 100 μM IBMX plus 5 μM ionomycin ($n = 7$). Protein secretion is low and almost constant over the whole stimulation period with ionomycin (A), db cAMP (B), forskolin (C), or IBMX (D). A higher protein secretion could be activated by the simultaneous addition of forskolin and ionomycin (F), i.e. by increasing $[Ca^{2+}]_i$ and $[cAMP]_i$ simultaneously. The higher rates and transient pattern of protein secretion characteristic for 5-HT-stimulated protein secretion could be mimicked by either the simultaneous application of IBMX and forskolin (E), by application of IBMX plus ionomycin (G), or by the simultaneous application of forskolin, IBMX and ionomycin (H).

resulted in lower rates of protein secretion. The effectiveness of the PDE inhibitor IBMX in increasing $[cAMP]_i$ is critically dependent on the basal adenylyl cyclase activity. This activity seems to be low in the central cells of the cockroach salivary gland, since the secretion of protein is only slight without additional adenylyl cyclase stimulation. This notion is supported by direct measurements of cAMP concentrations in locust salivary glands (Ali and Orchard, 1994). In this preparation, the application of IBMX alone increases glandular cAMP content only moderately and indeed much less than the simultaneous application of 5-HT and IBMX. The strong increase in protein secretion after the application of IBMX and forskolin to *P. americana* salivary glands indicates that IBMX inhibits PDE effectively. An increase in $[cAMP]_i$ after the addition of forskolin or the membrane-permeable cAMP-analogue db cAMP depends on PDE activity. A high basal activity of PDE would prevent an effective increase in $[cAMP]_i$ and could explain the low rates of protein secretion observed after application of db cAMP and forskolin without IBMX. It is also possible that PDE is stimulated only after the activation of adenylyl cyclase by forskolin. Such a scenario has been described by Heslop and Berridge (1980) for the blowfly salivary gland. In this tissue, 5-HT transiently increases $[cAMP]_i$ by stimulating adenylyl cyclase activity followed by stimulation of PDE activity.

We have found that cAMP-mediated protein secretion is augmented by an increase in $[Ca^{2+}]_i$. Protein secretion is increased by the simultaneous application of either forskolin or IBMX together with ionomycin, compared with the secretion elicited by any of these three substances alone. As discussed above, the lower effectiveness of either forskolin or IBMX to activate protein secretion might be attributable to either high basal PDE or low basal adenylyl cyclase activity. A Ca^{2+}-activated isoform of adenylyl cyclase could well explain our results. Such Ca^{2+}-activated isoforms of adenylyl cyclase have been found in mammals and *Drosophila* (Smit and Iyengar, 1998; Yovell et al., 1992). In the blowfly salivary gland, adenylyl cyclase activity also depends on the presence of Ca^{2+} (Heslop and Berridge, 1980).

We have tested the effects of the putative adenylyl cyclase inhibitor MDL 12,330 A on the rate of 5-HT-stimulated protein secretion to obtain further experimental evidence of an involvement of the cAMP pathway in the activation of protein secretion. We have found that MDL 12,330 A augmented rather than inhibited 5-HT-induced protein secretion. Heslop and Berridge (1980) report that this substance can effectively block 5-HT-induced cAMP production by inhibiting adenylyl cyclase in the *Calliphora* salivary gland. In cockroach salivary glands, MDL 12,330 A inhibits dopamine-stimulated fluid secretion (Gray et al., 1984), which is also thought to be activated by an increase in $[cAMP]_i$. We have confirmed the inhibitory effect on fluid secretion, indicating that MDL 12,330 A inhibits adenylyl cyclase activity in the acinar p-cells, which are responsible for fluid secretion. In contrast, MDL 12,330 A seems to activate adenylyl cyclase in the c-cells, thus increasing the rate of 5-HT-induced protein secretion. An adenylyl cyclase-activating effect of MDL 12,330 A on some adenylyl cyclase isoforms has been reported for the snail *Planorbarius corneus* (Ferretti et al., 1996). Furthermore, an inhibitory effect of MDL 12,330 A on PDE activity has been observed in ganglia of the leech *Hirudo medicinalis* and in some vertebrate tissues (Biondi et al., 1990; Hunt and Evans, 1980). This effect would also lead to an increase in $[cAMP]_i$ in the presence of MDL 12,330 A and could explain our observations.

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The effects of dopamine receptor agonists and antagonists on the secretory rate of cockroach (*Periplaneta americana*) salivary glands

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Abstract

The acinar salivary glands of the cockroach, *Periplaneta americana*, are innervated by dopaminergic and serotonergic nerve fibers. Serotonin stimulates the secretion of protein-rich saliva, whereas dopamine causes the production of protein-free saliva. This suggests that dopamine acts selectively on ion-transporting peripheral cells within the acini and the duct cells, and that serotonin acts on the protein-producing central cells of the acini. We have investigated the pharmacology of the dopamine-induced secretory activity of the salivary gland of *Periplaneta americana* by testing several dopamine receptor agonists and antagonists. The effects of dopamine can be mimicked by the non-selective dopamine receptor agonist 6,7-ADTN and, less effectively, by the vertebrate D1 receptor-selective agonist chloro-APB. The vertebrate D1 receptor-selective agonist SKF 38393 and vertebrate D2 receptor-selective agonist *R*(−)-TNPA were ineffective. *R*(+)-Lisuride induces a secretory response with a slower onset and a lower maximal response compared with dopamine-induced secretion. However, lisuride-stimulated glands continue secreting saliva, even after lisuride-washout. Dopamine-induced secretions can be blocked by the vertebrate dopamine receptor antagonists *cis*(*Z*)-flupenthixol, chlorpromazine, and *S*(+)-butaclamol. Our pharmacological data do not unequivocally indicate whether the dopamine receptors on the *Periplaneta* salivary glands belong to the D1 or D2 subfamily of dopamine receptors, but we can confirm that the pharmacology of invertebrate dopamine receptors is remarkably different from that of their vertebrate counterparts.

Keywords: Biogenic amine; Dopamine; G protein-coupled receptor; Insect; Salivary gland; Secretion

1. Introduction

Biogenic amines are important messenger molecules in the central nervous system and in peripheral organs of vertebrates and invertebrates (for recent reviews, see: Blenau and Baumann, 2001; Baumann et al., 2003). Physiologically, they can act as neurotransmitters, neuromodulators, or neurohormones. Serotonin (5-HT), dopamine, tyramine, octopamine, and histamine are amines that have been shown to exist in insects. The molecular and pharmacological characterization of amine receptors in insects is still in its infancy but is a prerequisite for the understanding of amine-dependent physiological processes. A well-established model system for studying the cellular actions of biogenic amines are the salivary glands of various insect species (for reviews, see: House, 1980; House and Ginsborg, 1985; Ali, 1997; Zimmermann and Walz, 2003).

The salivary glands in the cockroach *Periplaneta americana* are of the acinar type (Just and Walz, 1994). The acini consist of two secretory cell types, the peripheral and central cells. Experiments with isolated salivary glands have shown that stimulation with dopamine induces the secretion of a protein-free saliva, which is produced by the activity of the peripheral cells (Just and Walz, 1996). Serotonin induces the secretion of a protein-containing saliva by stimulating peripheral and central cells, whereby protein is secreted only from the central cells (Just and Walz, 1996). Salivary duct cells are also stimulated by dopamine (Lang and Walz,

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1999, 2001) and modify the primary saliva (Rietdorf et al., 2003). This behavior of isolated glands reflects their innervation pattern. Cockroach salivary glands and the morphologically similar salivary glands of the locust are innervated by dopaminergic and serotonergic nerve fibers (Whitehead, 1971; Davis, 1985, 1987; Gifford et al., 1991; Ali et al., 1993; Elia et al., 1994). A recent study by Baumann et al. (2002) has shown that, in *Periplaneta* salivary glands, the peripheral cells are innervated by both dopaminergic and serotonergic fibers, whereas the central cells are innervated only by serotonergic fibers.

Pharmacological data suggest that dopamine and serotonin stimulate locust salivary glands via specific receptors (Ali and Orchard, 1994), but the exact localization of these receptors on specific cell types within the salivary gland tissue is unknown. Although some information is available on the pharmacology of dopamine receptors in the salivary glands of the cockroach *Nauphoeta cinerea* (Grewe and Kebabian, 1982; House and Ginsborg, 1985; Evans and Green, 1990a,b, 1991) and of *Locusta migratoria* (Lafon-Cazal and Bockaert, 1984; Ali and Orchard, 1994; Keating and Orchard, 2001, 2004), little is known about the pharmacological properties of the dopamine receptor(s) mediating saliva secretion in *Periplaneta americana*. Here, we describe the pharmacological effects of several dopamine receptor agonists and antagonists on saliva secretion in *Periplaneta americana*, with experiments that supplement our current physiological and molecular characterization of secretory processes in insects.

2. Materials and methods

2.1. Animals

Cockroaches were taken from a colony of *Periplaneta americana* reared at 27 °C under a light:dark cycle of 12:12 h. The animals had free access to food and water. Only male adults aged between 3 and 6 weeks were used.

2.2. Tissue preparation and measurements of fluid secretion

Measurements of fluid secretion and the collection of saliva for protein determinations were carried out as described earlier (Just and Walz, 1996; Rietdorf et al., 2003). Briefly, animals were dissected in cockroach saline (160 mM NaCl, 10 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glucose, 10 mM Tris, pH 7.4) and, after removal of the reservoirs, the salivary glands and their ducts were transferred to a perfusion chamber divided into two compartments. The salivary glands were positioned in the first compartment (volume ~0.5 ml) and continuously perfused with oxygenated cockroach saline at a rate of about 0.5 ml/min, whereas the main salivary duct was carefully drawn across a dividing barrier and pinned to the base of the second compartment, which was filled with liquid paraffin. Secretion was stimulated by addition of drugs to the perfusate. Stimulation protocols for testing dopamine receptor agonists and antagonists are shown in Fig. 1. Saliva droplets were collected once per minute from the cut end of the main duct by using gel loader tips, and transferred to a second pool of liquid paraffin. Longitudinal and transverse diameters ($2a$ and $2b$, respectively) of the usually ellipsoid saliva droplets were measured by using a CCD video camera (Hamamatsu, Herrsching, Germany) attached to a LEICA MZAPO stereo microscope and an Argus-10 imaging processor (Hamamatsu, Herrsching, Germany). The volume (V) of a drop of saliva was calculated according to the equation $V = (4/3)\pi ab^2$.

Individual salivary glands exhibit some variability in their absolute secretory rates. In order to minimize gland-to-gland variation on experimental data, every gland was stimulated first with 1 μM dopamine for 10 min (control stimulation with dopamine). Because secretory rates usually rise during the first 3 min after onset of stimulation, mean secretory rates measured between min 4–10 during this control stimulation were defined as being 100%. All other secretion rates were normalized to this control rate. In the bar diagrams (Figs. 4 and 6), mean secretory rates measured between min 29–40 (agonists) and min 34–40 (antagonists), respectively, are shown. A final stimulation with 1 μM serotonin was used as a control for protein secretion (control stimulation with serotonin). In the text, values are given as mean ± SEM[SD/$\sqrt{n}$]. Data from individual glands were not used for the analysis when one of the following criteria applied: (1) the measurement was incomplete; (2) the rate of fluid secretion did not raise above 100 nl/min during the control stimulation with dopamine (min 1–10); (3) the secretory rate fell below 50% of the maximal value during the control stimulation with dopamine; (4) the secretory rate rose to more than 150% of the control stimulation with dopamine during the test stimulation. Statistic significance was determined using one-way analysis of variance (ANOVA, Kruskall–Wallis test followed by Dunn's multiple comparison test) with P values ≤ 0.05 considered significant. Data were analyzed and displayed using Prism 4.01 (Graph Pad Software, San Diego CA, USA, www.graphpad.com).

2.3. Protein assay

After volume determination, saliva samples were transferred to an Eppendorf tube filled with liquid paraffin and stored at −20 °C until the protein assay

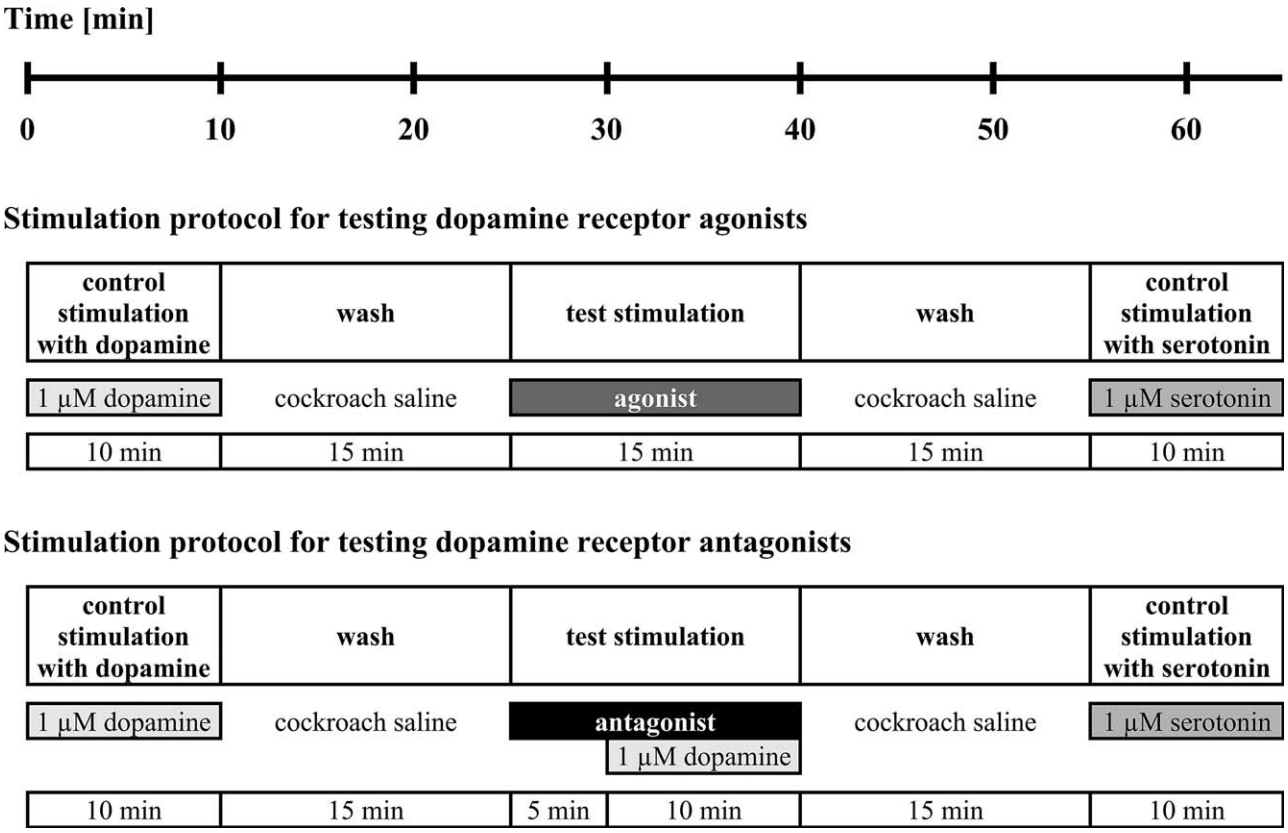

Fig. 1. Stimulation protocols for testing dopamine receptor agonists and antagonists. Each gland was first stimulated with 1 µM dopamine for 10 min (control stimulation with dopamine). After a dopamine washout (15 min), glands were stimulated with test substances (either agonist for 15 min or antagonist for 5 min, followed by a combination of both 1 µM dopamine and antagonist for 10 min). After a second washout (15 min), glands were stimulated with 1 µM serotonin for 10 min as a positive control for protein secretion (control stimulation with serotonin).

was performed. Protein content was measured by using a modified Bradford assay (Bradford, 1976) with Roti Nanoquant solution (Roth, Karlsruhe, Germany) as reagent according to the suppliers' instructions. Each droplet of saliva was transferred to one well of a 96-well microtiterplate and diluted with 50 µl H_2O. An aliquot of 200 µl of Roti Nanoquant working solution was added to each well. After 5 min, the absorption was measured using a Milenia Kinetic Analyzer (Diagnostic Products Corporation, Los Angeles, USA). For complete mixture of the samples, plates were shaken in the analyzer three times prior to measurement. Each microtiterplate was calibrated by using standards of the following concentrations of bovine serum albumin (BSA): 0, 20, 30, 40, 50, 60, 80, and 100 µg/ml. Since small amounts of liquid paraffin were transferred together with saliva droplets, 3 µl of liquid paraffin were added to each well containing a standard. Absorption of standards was measured together with the samples. The ratio of the absorption at 650/450 nm was used for analysis. A linear regression was calculated for the BSA standards and used for quantification of protein content in the samples. The lower detection limit was 10 µg/ml, corresponding to ~500 ng protein in a saliva sample, whereas the upper detection limit was 100 µg/ml, corresponding to ~5 µg protein in a saliva sample. When the staining of an individual 250 µl sample appeared to be darker than that of the 100 µg/ml BSA standard, the sample was diluted 1:1 with the respective components (125 µl sample + 25 µl H_2O + 100 µl Roti Nanoquant working solution) prior to measurement.

2.4. Chemicals and solutions

Dopamine (3-hydroxytyramine) HCl, serotonin (5-hydroxytryptamine) HCl, (±)-6-chloro-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzapine (chloro-APB, =(±)-SKF 82958) HBr, *R*(+)-lisuride hydrogen maleate, *R*(+)-SKF 38393 HCl, *R*(−)-2,10,11-trihydroxy-*N*-propyl-noraporphine (*R*(−)-TNPA) HBr, *S*(+)-butaclamol HCl, chlorpromazine HCl, and *cis*(*Z*)-flupenthixol 2HCl were from Sigma (Deisenhofen, Germany). (±)-2-Amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphtalene HBr (6,7-ADTN HBr) was from RBI (Cologne, Germany). Stock solutions were prepared in water for dopamine (10 mM), serotonin (10 mM), 6,7-ADTN (1 mM), *R*(+)-SKF 38393 (3 mM),

chlorpromazine (1 mM), and *cis*(*Z*)-flupenthixol (1 mM). *R*(+)-Lisuride (1 mM) was dissolved in 10% methanol. Chloro-APB (1 mM), *R*(−)-TNPA (1 mM), and *S*(+)-butaclamol (1 mM) were dissolved in 10% ethanol. All stock solutions were stored at −20 °C in small aliquots except those of 6,7-ADTN, *R*(−)-TNPA, and *S*(+)-butaclamol, which were prepared freshly each day. Drugs were diluted in cockroach saline to attain their final concentrations and added via the perfusate. Ethanol from the *S*(+)-butaclamol stock solution did not affect secretory rates (five glands tested).

3. Results

3.1. Effects of dopamine and serotonin on the secretory response

Isolated salivary glands perfused with oxygenated cockroach saline respond to agonist stimulation for longer than 70 min. In the absence of agonist, secretory responses from isolated glands were negligible. Stimulation with 1 μM dopamine increased secretion to a rate of 556.8 ± 55.1 nl/min reversibly ($n = 17$, range: 100.8–982.3 nl/min). Secretion rates measured between min 4–10 (control stimulation with dopamine) were defined as 100% for each individual gland. During a second stimulation with 1 μM dopamine (test stimulation), the glands secreted at a lower rate of 79.9 ± 6.3% (min 29–40, Fig. 2A). The dose–response curve for the dopamine-induced saliva secretion in *Periplaneta americana* was as established previously: half-maximal secretory responses were obtained by using ~110 nM dopamine (Just and Walz, 1996). Saliva secreted upon stimulation with dopamine was always protein-free (Table 1, and Just and Walz, 1996). When 1 μM serotonin was applied during the test stimulation, secretory rates reached only 55.5 ± 4.4% (min 29–40, Fig. 2B), and serotonin-induced saliva contained protein (Table 1, and Just and Walz, 1996).

3.2. Effects of dopamine receptor agonists

Several synthetic dopamine receptor agonists were tested for their ability to induce secretion. The agonists were applied for 15 min, and their effects were compared with those obtained with 1 μM dopamine (Figs. 3 and 4). Two non-selective dopamine receptor agonists, 6,7-ADTN and *R*(+)-lisuride, and the D1 receptor-selective agonist chloro-APB stimulated saliva secretion. ADTN seemed to act as an effective agonist, evoking a response similar in time course and maximal effect to that evoked by 1 μM dopamine (Fig. 3A). The lisuride-induced secretory response was slower in onset and reached a lower maximal response compared with dopamine-induced secretion (Fig. 3B). Lisuride-stimulated glands continued secreting saliva even after lisuride-washout. Chloro-APB was slightly less effective than dopamine (Figs. 3C and 4). Saliva secreted after stimulation with ADTN, lisuride, or chloro-APB had no measurable protein content (Table 1) arguing against serotonin receptors being activated by these drugs. In experiments with the D1 receptor-selective agonist *R*(+)-SKF 38393 as well as with the D2 receptor-selective agonist *R*(−)-TNPA at concentrations of 1 and 10 μM, no or only minute secretory responses could be detected (Figs. 3D, E and 4).

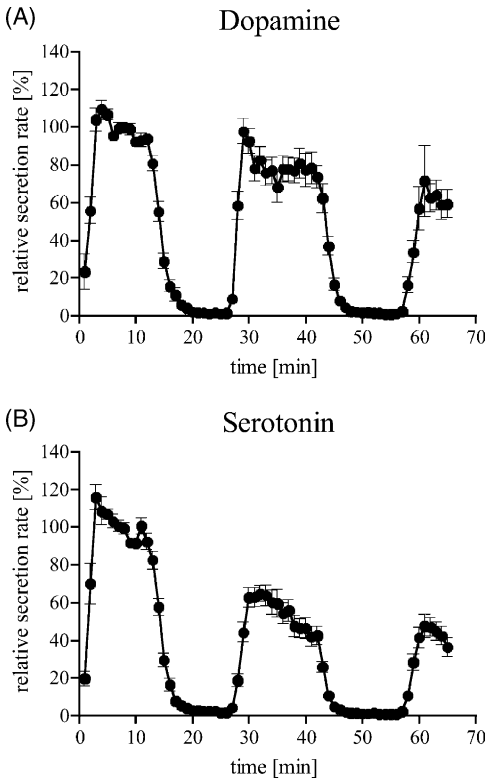

Fig. 2. Relative rates of secretion upon stimulation with 1 μM dopamine (A) and 1 μM serotonin (B). Individual data points are means ± SEM. (A) $n = 17$; (B) $n = 15$.

Table 1
Agonist-stimulated protein secretion. Only 1 μM serotonin induced protein secretion. Data are means ± SEM (n = number of glands tested)

Agonist	Protein secretion (μg/ml) (mean ± SEM)	n
Dopamine (1 μM)	0.02 ± 0.0025	16
Serotonin (1 μM)	2.19 ± 0.229	15
6,7-ADTN (1 μM)	0.1 ± 0.1	10
R(+)-Lisuride (1 μM)	0 ± 0	10
Chloro-APB (1 μM)	0 ± 0	10
Chloro-APB (10 μM)	0 ± 0	5
R(−)-TNPA (10 μM)	0 ± 0	6

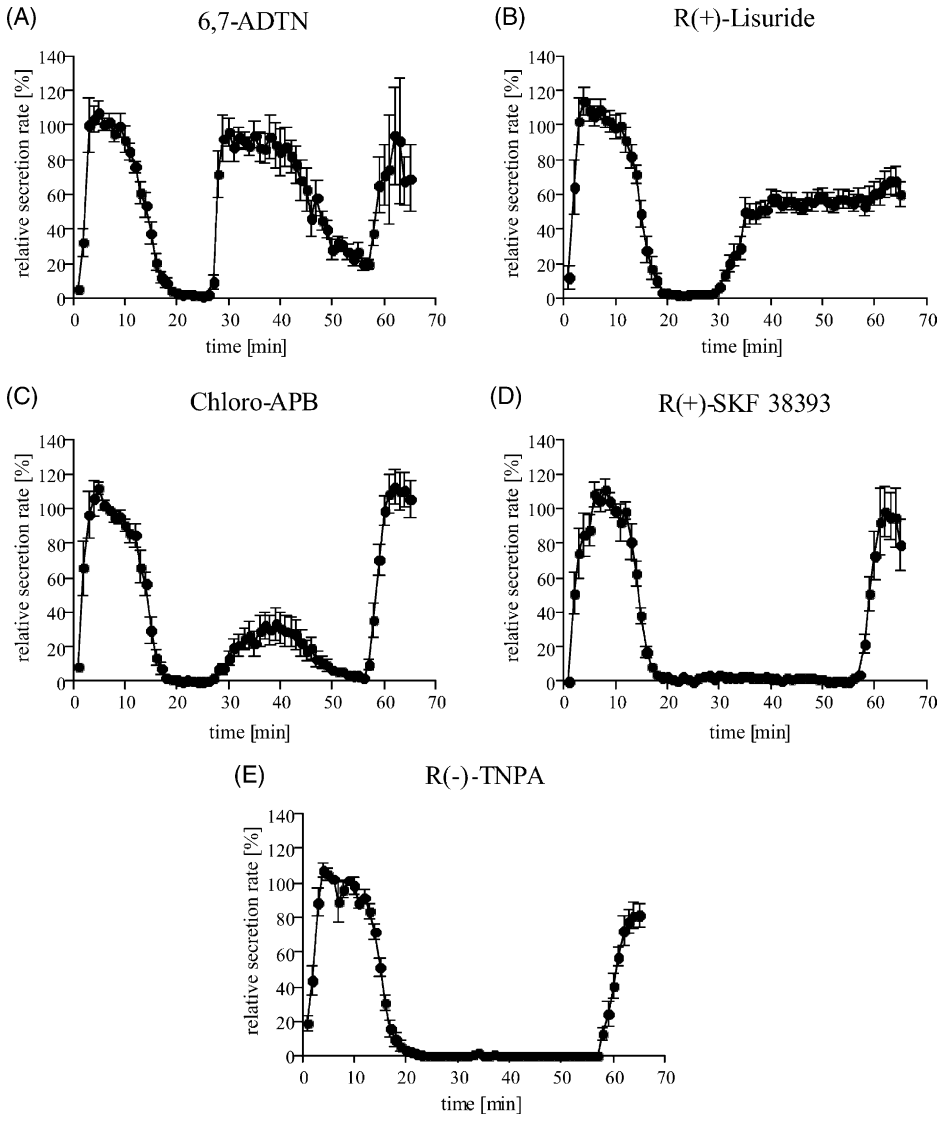

Fig. 3. Relative rates of secretion upon stimulation with various dopamine receptor agonists. Saliva secretion was induced by (A) the non-selective dopamine receptor agonist 6,7-ADTN (1 μM, $n = 10$), (B) the non-selective dopamine receptor agonist *R*(+)-lisuride (1 μM, $n = 10$), (C) the D1-like dopamine receptor agonist chloro-APB (1 μM, $n = 10$), (D) the D1-like dopamine receptor agonist *R*(+)-SKF 38393 (1 μM, $n = 10$), or (E) the D2-like dopamine receptor agonist *R*(−)-TNPA (1 μM, $n = 9$). Individual data points are means ± SEM.

3.3. *Effects of dopamine receptor antagonists on dopamine-induced saliva secretion*

Using a protocol in which putative antagonists were applied together with 1 μM dopamine (Fig. 1), we tested the ability of two non-selective dopamine receptor antagonists, *cis*(*Z*)-flupenthixol and *S*(+)-butaclamol, and the D2 receptor-selective antagonist chlorpromazine to block dopamine-induced saliva secretion. All three antagonists blocked dopamine-induced secretion to a similar degree when tested at the same concentrations of either 10^{-6} or 10^{-5} M, respectively (Figs. 5 and 6). In addition, the serotonin-induced secretory response (control stimulation with serotonin, min 59–65) was also significantly reduced after treatment with either chlorpromazine or butaclamol and washout (18.6 ± 4.3% and 5.5 ± 2.2% following pre-treatment with 10 μM chlorpromazine and 10 μM butaclamol, respectively) in comparison with experiments where only dopamine was applied during the test stimulation (57.8 ± 8.3%). This indicates that butaclamol and chlorpromazine may also act as serotonin receptor antagonists, but this has to be tested more directly in future experiments.

4. Discussion

In this study, we have evaluated the pharmacological properties of the dopamine receptor(s) involved in

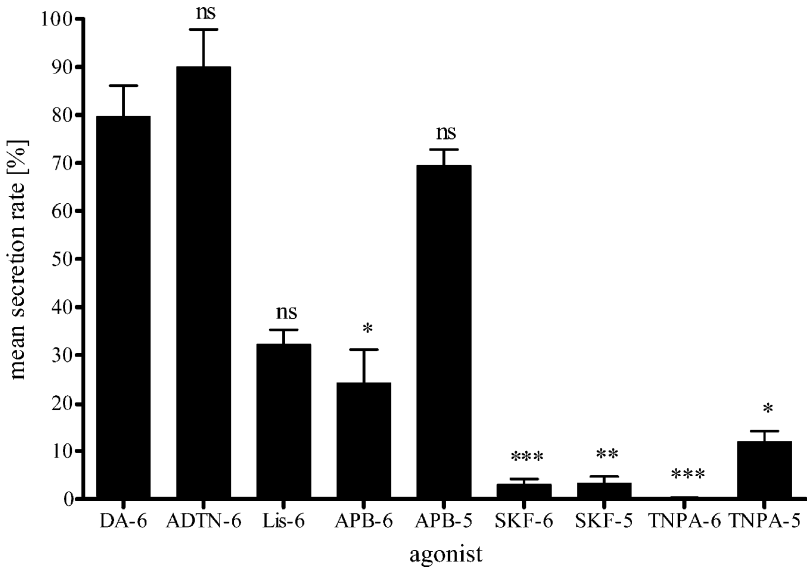

Fig. 4. Mean secretory rates upon stimulation with various dopamine receptor agonists. Bar chart summarizing the mean secretory rates (min 29–40) upon stimulation with 1 μM dopamine (DA-6, $n = 17$), 1 μM ADTN (ADTN-6, $n = 10$), 1 μM lisuride (Lis-6, $n = 10$), 1 μM chloro-APB (APB-6, $n = 10$), 10 μM chloro-APB (APB-5, $n = 5$), 1 μM SKF 38393 (SKF-6, $n = 10$), 10 μM SKF 38393 (SKF-5, $n = 5$), 1 μM *R*(−)-TNPA (TNPA-6, $n = 9$), and 10 μM *R*(−)-TNPA (TNPA-5, $n = 6$). Individual data points are means ± SEM. Secretory rates upon stimulation with agonists were statistically compared with secretory rate upon stimulation with 1 μM dopamine (Dunn's multiple comparison test; ns $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

mediating the stimulatory effect of dopamine on saliva secretion in *Periplaneta americana*. Although our findings do not yet define which subtype(s) of dopamine receptor are present in the salivary gland of *Periplaneta americana*, they impose constraints on the nature of the receptor subtypes that might be expected, and they contribute to the comparative pharmacology of amine receptors in insects.

The catecholamine dopamine is an important regulator of physiological and behavioral functions. In vertebrates, dopamine binds to two subfamilies of dopamine receptors: D1- and D2-(like) receptors (Kebabian and Calne, 1979). These receptors belong to the family of G protein-coupled receptors and differ in their pharmacological and biochemical properties. D1 and D5 receptors constitute the D1 subfamily and activate adenylyl cyclase, whereas members of the D2 subfamily, i.e. the D2, D3, and D4 receptors, either inhibit adenylyl cyclase or couple to different intracellular second messenger systems (for reviews, see: Seeman and Van Tol, 1994; Missale et al., 1998; Callier et al., 2003). Dopamine is also an important neuroactive substance in the nervous system of a variety of insect species including the cockroach *Periplaneta americana* (for reviews, see: Downer, 1990; Blenau and Baumann, 2001; Roeder, 2002).

In salivary glands of *Nauphoeta cinerea* (House and Ginsborg, 1979, 1985; House, 1980) and *Periplaneta americana* (this study), the application of dopamine or the agonist 6,7-ADTN induces the secretion of a protein-free saliva. 6,7-ATDN, a naphthalene, has also been shown to act as a potent dopamine receptor agonist in other insect systems: it increases cAMP production in the salivary glands of *Nauphoeta cinerea* (Grewe and Kebabian, 1982) and *Locusta migratoria* (Lafon-Cazal and Bockaert, 1984), in the brain of *Periplaneta americana* (Orr et al., 1987), and via heterologously expressed dopamine D1 receptors of *Drosophila melanogaster* (Sugamori et al., 1995) and the honeybee *Apis mellifera* (Blenau et al., 1998; Mustard et al., 2003). Both dopamine and 6,7-ADTN generate inward currents in the soma of a common inhibitory motoneuron in the prothoracic ganglion of adult male cockroaches (Davis and Pitman, 1991) and reduce sucrose responsiveness in honeybees (Scheiner et al., 2002). In both vertebrate and invertebrate tissues, ADTN activates D1-like receptors coupled to adenylyl cyclase, but is not selective and also binds to D2-like receptors (Hearn et al., 2002). Therefore, the activity of this compound does not differentiate between D1- and D2-like receptors.

R(+)-Lisuride, a non-hallucinogenic congener of LSD, induces a secretory response with a slower onset and a lower maximal response compared with dopamine-induced secretion. Furthermore, lisuride-induced secretion is not reversible, at least not during the time course of our experiments. In vertebrate systems, lisuride has long been known to have reduced efficacy relative to dopamine and acts as a partial agonist (for a review, see: Pulvirenti and Koob, 1994). Lisuride also

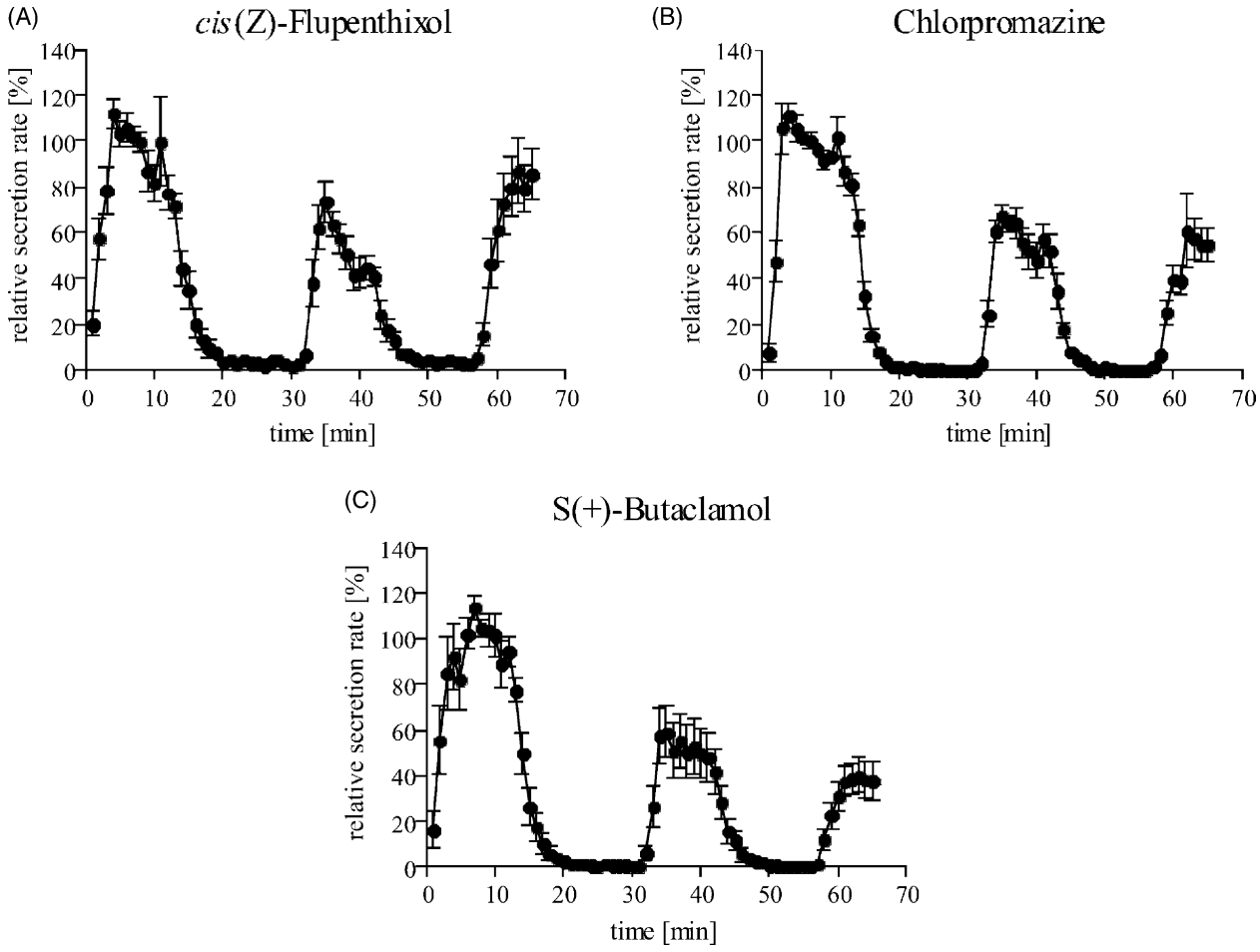

Fig. 5. Inhibition of dopamine-induced secretion by dopamine receptor antagonists. (A) The non-selective dopamine receptor antagonist *cis*(*Z*)-flupenthixol (1 μM, $n = 12$), (B) the D2-like dopamine receptor antagonist chlorpromazine (1 μM, $n = 10$), and (C) the non-selective dopamine receptor antagonist *S*(+)-butaclamol (1 μM, $n = 10$) were tested. Individual data points are means ± SEM.

shows high affinity binding to dopamine (Blenau et al., 1995a, 1998) and serotonin (Blenau et al., 1995b) receptors of other insects.

Chloro-APB (SKF 82958) can also mimic the effect of dopamine in evoking salivary secretion in *Periplaneta americana* and *Locusta migratoria* (Keating and Orchard, 2004). APB is a full agonist at mammalian D1 receptors and also at D1 receptors of *Drosophila* (Feng et al., 1996; Reale et al., 1997) and the honeybee (Mustard et al., 2003). Thus, the dopamine receptors in the *Periplaneta* salivary gland might belong to the D1 subfamily of dopamine receptors.

On the other hand, the benzazepine *R*(+)-SKF 38393 (in concentrations up to 10 μM) neither activates saliva secretion in *Periplaneta* nor stimulates the dopamine-sensitive adenylyl cyclase in the brain of this insect (Orr et al., 1987). This substance is a strongly selective dopamine D1 receptor agonist in vertebrates (for reviews, see: Seeman and Van Tol, 1994; Missale et al., 1998) but has been shown to be a poor agonist at D1 receptors of insects (Gotzes et al., 1994; Sugamori et al., 1995; Feng et al., 1996; Blenau et al., 1998; Mustard et al., 2003). In the salivary gland of *Nauphoeta cinerea*, SKF 38393 elicits a secretory response but with a threshold concentration as high as 40 μM (Evans and Green, 1991).

The aporphine alkaloid *R*(−)-TNPA is a D2 receptor-selective agonist in vertebrates. TNPA at high concentration (10 μM) induces only a slight secretory response in *Periplaneta* salivary glands and was ineffective in locust salivary glands (Keating and Orchard, 2004).

In protostomes, the thioxanthine *cis*(*Z*)-flupenthixol and the butyrophenon *S*(+)-butaclamol have been shown to be effective antagonists on both D1-like (Gotzes et al., 1994; Sugamori et al., 1995; Blenau et al., 1998; Mustard et al., 2003) and D2-like (Hearn et al., 2002; Suo et al., 2003) receptors, as they are in vertebrates. Flupenthixol and butaclamol are also effective antagonists on the dopamine receptors of *Periplaneta* salivary glands, but as these compounds are not selective for either D1-like or D2-like receptors, they cannot be used to distinguish the receptor subfamily. In

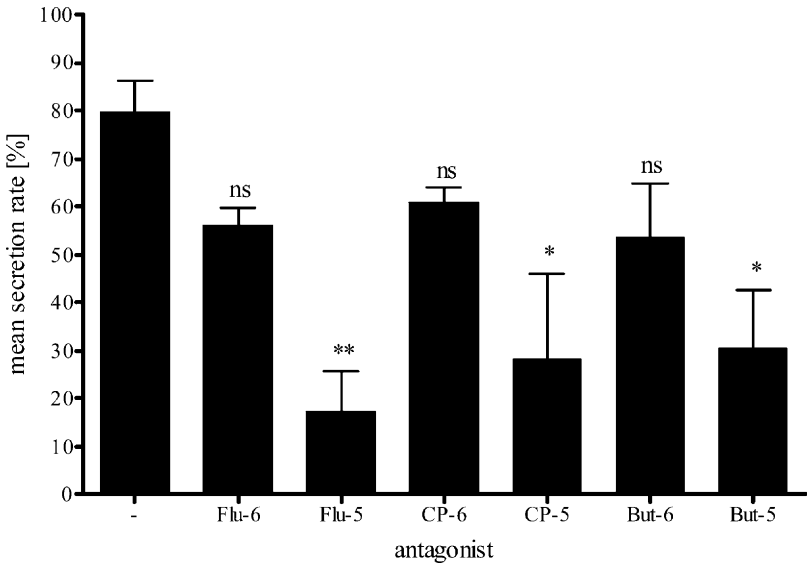

Fig. 6. Inhibition of dopamine-induced secretion by dopamine receptor antagonists. Bar chart summarizing the mean dopamine-induced secretory rates in the presence of (min 34–40) no antagonist (–, $n = 17$), 1 μM flupenthixol (Flu-6, $n = 12$), 10 μM flupenthixol (Flu-5, $n = 5$), 1 μM chlorpromazine (CP-6, $n = 10$), 10 μM chlorpromazine (CP-5, $n = 5$), 1 μM butaclamol (But-6, $n = 10$), and 10 μM butaclamol (But-5, $n = 5$). All three dopamine receptor antagonists caused a dose-dependent suppression of dopamine-induced secretion. Individual data points are means ± SEM. Secretory rates in the presence of antagonists were statistically compared with secretory rate in the absence of antagonist (Dunn's multiple comparison test; ns $P > 0.05$; * $P < 0.05$; ** $P < 0.01$).

Periplaneta americana, both substances are also effective in inhibiting the dopamine-sensitive adenylyl cyclase in the brain (Orr et al., 1987) and in the corpus cardiacum (Gole et al., 1987), displacing [^{3}H]pifluthixol binding in brain homogenates (Notman and Downer, 1987) and blocking the actions of dopamine on the identified inhibitory motoneuron D_3 (Davis and Pitman, 1991). Flupenthixol also blocks glandular responses to nerve stimulation and dopamine application in *Nauphoeta cinerea* (House and Ginsborg, 1979, 1985; House, 1980). In the *Periplaneta* salivary gland, there is also some preliminary evidence that *S*(+)-butaclamol acts as a serotonin receptor antagonist, as has been shown for serotonin receptors of *Drosophila* (Saudou et al., 1992).

The third antagonist shown to be active in blocking the dopamine response in *Periplaneta* salivary glands was the phenothiazine compound chlorpromazine. This anti-psychotic drug shows some preference for D2 over D1 receptors in vertebrates (Seeman and Van Tol, 1994), but the phenothiazines are notorious for their wide spectrum of pharmacological and biochemical effects. In insects, chlorpromazine binds with high affinity to D1-like receptors of *Drosophila* (Sugamori et al., 1995) and *Apis* (Blenau et al., 1998; Mustard et al., 2003) and to tyramine receptors of various species (Vanden Broeck et al., 1995; Robb et al., 1994; Ohta et al., 2003). Thus, chlorpromazine cannot be considered as a specific D2 receptor antagonist.

Our pharmacological data do not unequivocally indicate whether the dopamine receptors on the *Periplaneta* salivary glands belong to the D1 or D2 subfamily of dopamine receptors, but we can confirm that the pharmacology of invertebrate dopamine receptors is remarkably different from that of their vertebrate counterparts (for reviews, see: Downer, 1990; Blenau and Baumann, 2001; Roeder, 2002).

D1-like dopamine receptors are positively coupled to the enzyme adenylyl cyclase, whereas D2-like dopamine receptors either inhibit adenylyl cyclase or are coupled to different intracellular second messenger systems. Cyclic AMP in the bathing solution of isolated salivary glands of *Nauphoeta cinerea* causes a dose-dependent secretory response but fails to hyperpolarize the acinar cell membrane potential as dopamine does (Gray et al., 1984). Application of high doses (10 mM) of cAMP or dibuteryl-cAMP leads to saliva secretion (secretion rate only ~10% compared with the secretion induced by 1 μM dopamine) in ~50% of the *Periplaneta* salivary glands tested (Just and Rietdorf, personal communication). Dopamine has been shown to increase intracellular cAMP levels ($[cAMP]_i$) in the salivary glands of *Nauphoeta cinerea* (Grewe and Kebabian, 1982) and *Locusta migratoria* (Lafon-Cazal and Bockaert, 1984; Ali and Orchard, 1994), but unfortunately, it remains unknown in which cell type levels of cAMP rose. Although stimulation of cAMP production after dopamine application has not yet been shown directly for salivary glands of *Periplaneta americana*, these results strongly indicate the presence of D1-like dopamine receptors. We should also keep in mind that dopamine evokes a reversible dose-dependent increase in $[Ca^{2+}]_i$ in the salivary duct cells

(Lang and Walz, 1999). We perceive three possible explanations for these findings: (1) Two (or more) different receptor subtypes are expressed in the salivary gland, with one subtype causing an increase in $[cAMP]_i$ of the acinar cells and fluid secretion and a second subtype producing an increase in $[Ca^{2+}]_i$. Such a scenario has been suggested previously by Gray et al. (1984) for *Nauphoeta cinerea*. (2) Only one dopamine receptor subtype is expressed in the salivary gland complex but it is coupled to multiple second-messenger pathways. (3) Only one receptor subtype is expressed, and either the increase in $[cAMP]_i$ or the observed increase in $[Ca^{2+}]_i$ after stimulation with dopamine is a secondary effect. Such a "one receptor hypothesis" has been suggested by Evans and Green (1990a, b, 1991), who have found that a range of receptor ligands exhibit similar absolute and rank order potencies in blocking either the secretory response (which apparently uses cAMP as a second messenger) or the acinar cell hyperpolarization (which apparently involves Ca^{2+} mobilization) in *Nauphoeta cinerea*. The final decision regarding which type(s) of dopamine receptor is (are) present in the salivary gland of *Periplanta americana* awaits its (their) molecular identification.

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Charakterisierung des *ebony*-Gens der Schabe *Periplaneta americana*

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Molecular Characterization of the *ebony* Gene From the American Cockroach, *Periplaneta americana*

Wolfgang Blenau[1]* and Arnd Baumann[2]

Biogenic amines are an important class of primary messengers in the central (CNS) and peripheral nervous systems and in peripheral organs. These substances regulate and modulate many physiological and behavioral processes. Various inactivation mechanisms for these substances exist to terminate biogenic amine-mediated signal transduction. In vertebrates, the enzymes monoamine oxidase and/or catechol-*O*-methyl-transferase are involved in these processes. In insects, however, in which both enzymes are low in abundance or absent, biogenic amines are inactivated mainly by *N*-acetylation or *O*-sulphation. In *Drosophila,* β-alanyl conjugation mediated by the Ebony protein has recently been shown to be a novel and alternative pathway for biogenic amine inactivation. Here, we report the cloning of ebony cDNA (Pea*ebony*) from a brain-specific cDNA library of the cockroach *Periplaneta americana*. The open reading frame encodes a protein of 860 amino acid residues (PeaEbony). The PeaEbony polypeptide shares homology to Ebony sequences from *Anopheles gambiae, Apis mellifera,* and *Drosophila melanogaster*. In addition, PeaEbony exhibits sequence similarity to a family of microbial non-ribosomal peptide synthetases. The mRNA encoding PeaEbony is highly expressed in the cockroach brain and to a lesser extent in the salivary glands. PeaEbony is, therefore, probably involved in the inactivation of various biogenic amines through β-alanyl conjugation in the cockroach CNS. Since the salivary glands in *Periplaneta* are innervated by dopaminergic and serotonergic neurons, PeaEbony probably also biochemically modifies dopamine and serotonin in these acinar glands. Arch. Insect Biochem. Physiol. 59:184–195, 2005.

KEYWORDS: biogenic amines; dopamine; neurotransmitter inactivation; non-ribosomal peptide synthetase; serotonin; signal transduction

INTRODUCTION

Biogenic amines are important primary messengers in insects in which they act as neurotransmitters, neuromodulators, and neurohormones (Evans, 1980; Downer, 1990; Blenau and Baumann, 2001, 2003; Roeder et al., 2003). They control and modulate the activity of neurons (for a review, see Bicker and Menzel, 1989), endocrine and exocrine secretion (Just and Walz, 1996; Marg et al., 2004), the contraction properties of muscles, and the generation of motor patterns (Claassen and Kammer, 1986), and have also been shown to be involved in learning and the formation of memory (Schwaerzel et al., 2003; for reviews, see Meller and Davis, 1996; Hammer, 1997). Biogenic amines mediate these diverse cellular and physiological effects by binding to specific membrane proteins that primarily belong to the superfamily of G-protein coupled receptors (for a review, see Blenau and Baumann, 2001). In contrast to our knowledge of biogenic-amine-activated signaling cascades, relatively little is known about the mechanism(s) involved in the termination of biogenic amine activity. The main mechanisms of biogenic amine inactivation are: (1) re-uptake into the releasing cell by amine-specific

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transporters and (2) degradation by enzymes and/or biochemical conjugation. Recently, a family of Na^+/Cl^--dependent transport proteins with functional resemblance to mammalian transporters has been described in insects (Caveney and Donly, 2002). Compared with mammals, enzymes metabolizing biogenic amines, i.e., monoamine oxidase (MAO) and catechol-*O*-methyl-transferase (COMT), play a minor role, if any, in the insect nervous system (for a review, see Sloley, 2004). Here, the inactivation of biogenic amines is achieved by alternative metabolic pathways such as *N*-acetylation, *N*-methylation, and *O*-sulphation (for reviews, see Wright, 1987; Vaughan, 1988; Downer, 1990; Roeder et al., 2003). In addition, the β-alanyl conjugation of biogenic amines by Ebony proteins is a novel pathway now known to be involved in the inactivation of biogenic amines in insects (Pérez et al., 2002, 2004; Richardt et al., 2003).

In *Drosophila*, mutations of the *ebony* gene (Bridges and Morgan, 1923) cause a number of phenotypes, ranging from a dark cuticle (Brehme, 1941) to behavioral defects (Jacobs, 1978; Newby and Jackson, 1991; Baier et al., 2002). Biochemically, the cuticle phenotype is caused by a loss of β-alanyl-dopamine synthesis in mutant flies (Jacobs and Brubaker, 1963; Hodgetts and Konopka, 1973). These results have led to the assumption that *ebony* is the structural gene for β-alanyl-dopamine synthetase (Wright, 1987). In addition to sclerotization and melanization of the cuticle, mutations of the *ebony* gene affect visual signal transduction. The electroretinogram (ERG) of certain *ebony* mutants lacks the on- and off-transients of the light response (Hotta and Benzer, 1969; Heisenberg, 1971). Characterization of the *ebony* locus (Caizzi et al., 1987) and cloning of the *ebony* gene (Hovemann et al., 1998) from *Drosophila* has shown that the polypeptide shares high homology with microbial non-ribosomal peptide synthetases (NRPSs; Hovemann et al., 1998). The Ebony protein has been immunohistochemically identified in glia cells surrounding the photoreceptor terminals in the lamina and the distal medulla of the fruitfly (Richardt et al., 2002). This result is surprising because (1) histamine and not dopamine is released from photoreceptor terminals in the compound eye of insects (Hardie, 1987), and (2) dopaminergic neurons are absent from the lamina of *Drosophila* (Nässel et al., 1988). This contradiction, however, has been solved by biochemical experiments showing that *ebony* mutants have reduced histamine content in the head (Borycz et al., 2002) and are unable to convert histamine into its β-alanine conjugate, carcinine (Borycz et al., 2002). In vitro assays with heterologously expressed and purified *Drosophila* Ebony protein have shown that the activation and binding of β-alanine occurs in a peptide-synthetase-like manner. Furthermore, enzymatic activity has been observed not only with dopamine but also with histamine and other biogenic amines as substrates (Richardt et al., 2003).

Here, we describe the molecular cloning of a cDNA that we have prepared from the cockroach *Periplaneta americana* and that displays high homology to the *Drosophila ebony* gene. A high degree of sequence conservation has also been found to unique genes in the completely sequenced genomes of *Anopheles gambiae* and *Apis mellifera*. Notably, signature sequence motives of adenylation and thiolation domains of NRPSs are well conserved. Reverse transcription/polymerase chain reaction (RT-PCR) experiments have shown that *Periplaneta ebony* mRNA is highly expressed in the brain and to a lesser extent in the salivary glands of the cockroach. Since these tissues are known to contain significant amounts of biogenic amines, the PeaEbony enzyme is a likely candidate for participating in the inactivation of biogenic amines in this species.

MATERIALS AND METHODS

Materials

Radiolabeled [α-^{32}P]dCTP (specific activity, >3,000 Ci/mmol) was from Amersham Biosciences (Freiburg, Germany). Nylon membranes were from Qiagen (Hilden, Germany).

Isolation of cDNA Clones and Sequencing

Poly(A)$^+$ RNA was isolated from *Periplaneta americana* brains by using the Micro-FastTrack™ 2.0

Kit (Invitrogen, Karlsruhe, Germany). A cDNA library was constructed in λ -ZAP®II vector (Stratagene, Heidelburg, Germany) with oligo dT as a primer. We screened 1×10^6 recombinant λ-phages of the cDNA library with a fragment of the *Periplaneta ebony* gene (*ebony*-G13) as a probe. Hybridization was performed as described previously (Blenau et al., 1998). Plasmid DNA of positive clones was isolated following an in vivo excision protocol (Stratagene). Subcloning of restriction fragments was performed in the pBluescript II SK(–) vector (Stratagene) by standard cloning techniques (Sambrook et al., 2001). One cDNA clone (*ebony*-12) was completely sequenced on both strands. Sequencing was performed with the thermo sequenase fluorescent-labeled primer cycle sequencing kit (Amersham Biosciences) and the LICOR electrophoresis system (MWG Biotech). The nucleotide sequence of Pea*ebony* has been submitted to the EBI database (accession no. AJ865468).

Multiple Sequence Alignment and Phylogenetic Analysis

Amino acid sequences used for phylogenetic analysis were identified by protein-protein BLAST searches of the NCBI databases (BLASTP, NCBI database) with PeaEbony as "bait." Multiple sequence alignments of the complete amino acid sequences were performed with BioEdit (version 5.0.9; Hall, 1999). The genetic distance between sequences was calculated with ClustalX (version 1.81; Thompson et al., 1997) by using the Gonnet series protein weight matrix option. A neighbor joining tree was constructed with ClustalX by using 1,000-fold bootstrap re-sampling, and the resulting tree was displayed graphically by TreeView (Page, 1996).

RT-PCR Technique

Total RNA was isolated from the brain, salivary glands, Malpighian tubules, midgut, and muscle of adult male cockroach with TRIzol reagent (Invitrogen). The samples were digested with either DNase 1 (Ambion, Huntingdon, UK) to avoid genomic DNA contamination or DNase 1 and RNase Cocktail (Ambion) for negative controls. Pea*ebony*-specific fragments were amplified from 200 ng of total RNA by the SuperScript One-Step RT-PCR with Platinum *Taq* System (Invitrogen). The sense primer was 5′-GGAACAAGGGCAT GTTGAG-3′ and the antisense primer was 5′-GAGCAATGGGATGCTGTCC-3′. Cycling conditions were as follows: cDNA synthesis and pre-denaturation: 1 cycle of 50°C for 30 min, 94°C for 2 min; PCR amplifications: 35 cycles of 94°C for 40 s, 58°C for 1 min, 72°C for 1 min; final extension: 1 cycle of 72°C for 10 min.

RESULTS

A full-length cDNA clone (Pea*ebony*) was isolated by screening a λ-ZAP®II cDNA library constructed from *Periplaneta americana* brains. The Pea*ebony* cDNA (Fig. 1) consists of 2,793 bp and contains an open reading frame (ORF) of 2,583 bp. Stop codons are found in all three reading frames preceding the ATG initiation codon (position 46–48, Fig. 1). The 3′ non-coding region of Pea*ebony* consists of 210 nucleotides and is terminated by a poly(dA) tail of 8 residues. A polyadenylation signal (AATAAA; position 2,765–2,770) precedes the poly(dA) tail. The open reading frame of the gene encodes a polypeptide (PeaEbony) consisting of 860 amino acid residues with a calculated molecular weight of 95.5 kDa. Two consensus sites for phosphorylation by cAMP/cGMP-dependent protein kinase are present at serine 648 and 652. In addition, a total of 11 consensus sites for phosphorylation by protein kinase C are located at $Ser_{89/351/643/648/850/856}$ and $Thr_{486/527/541/695/736}$. Whether phosphorylation of any of these sites is necessary for enzymatic activity remains to be tested.

Fig. 1. Nucleotide and deduced amino acid sequence of the *ebony* gene from *Periplaneta americana*. Numbering of nucleotides starts with (+1) for the adenine of the translation initiation codon (ATG) and is given on the right of each line. The position of the deduced amino acids is also given on the right. The initiation codon (ATG) of the open reading frame and the stop codon (TAA) are given in bold letters and underlined. Peptide synthetase signature sequences are underlined.

```
GCGTGCTTCGCTTCGATCTGACTGACTGACGGACTGTGAGTAGTCATGGGTTCGATCCCCACATTGTCAATCCTGCGCGGCGACCCGCGC   45
                                               M  G  S  I  P  T  L  S  I  L  R  G  D  P  R    15
CCCCTGGACAGCGAGGCGTCGCTGCACAGACTGCTGGAGCGCGCGGCGGGCGCCTCGGCGCAGCAGACGGCGCTCCTGTTCCACAACCAG  135
 P  L  D  S  E  A  S  L  H  R  L  L  E  R  A  A  G  A  S  A  Q  Q  T  A  L  L  F  H  N  Q    45
AGCACGAGCTTCGGCGCGCTGGACGCCGCCGCCAGCCGCCTGGCCCGCGCCCTGGTGAGGCGCGCGCGCAACGTGGGCGCTCGCCCCAAC  225
 S  T  S  F  G  A  L  D  A  A  A  S  R  L  A  R  A  L  V  R  R  A  R  N  V  G  A  R  P  N    75
AGCGACGGGGACTTCCTGGTGGCCGTGTGCATGGAGCCCAGCGAGCGCCTCGTGGTGGCGCTGCTCGCCGTGTGGAAGGCGGGCGCCGCC  315
 S  D  G  D  F  L  V  A  V  C  M  E  P  S  E  R  L  V  V  A  L  L  A  V  W  K  A  G  A  A   105
TACCTGCCGCTGGACCCCGCCTTCCCCCAAGCCCGCGTGGCCCACATCCTGGGCGAGGCGCGCCCCGTGCTCGTCCTTGCCGAGAAGGAA  405
 Y  L  P  L  D  P  A  F  P  Q  A  R  V  A  H  I  L  G  E  A  R  P  V  L  V  L  A  E  K  E   135
GACCCTGAGCTGTTCAGCGACGCCGCTCTGTATGAGGATTTGCGCCGCGAGGCCGCGGACTTGTCCTCCGAGCCCTTGCCCGACGCCGAG  495
 D  P  E  L  F  S  D  A  A  L  Y  E  D  L  R  R  E  A  A  D  L  S  S  E  P  L  P  D  A  E   165
ACGCTGCCGGGCTGCGCCCCGACACTGGCGTTGGTGTTGTACACTTCCGGCAGCACGGGCGTGCCCAAGGGCGTGCGTCTACCTCACGCG  585
 T  L  P  G  C  A  P  T  L  A  L  V  L  Y  T  S  G  S  T  G  V  P  K  G  V  R  L  P  H  A   195
GTAGTCCTGAACAGACTTCGCTGGCAGTGGCGCACGTTCCCGTACGGCCCGCAGGAGCGCGTCTGCGTCTTCAAGACGGCCCTCACGTTC  675
 V  V  L  N  R  L  R  W  Q  W  R  T  F  P  Y  G  P  Q  E  R  V  C  V  F  K  T  A  L  T  F   225
GTGGACTCGGTGGGCGAGTTATGGGGACCCCTGCTGCAGGAGCGCAGCGTGCTCGTCGTCCCTCGCGAGGTCACCAAGGACCCCGAACGT  765
 V  D  S  V  G  E  L  W  G  P  L  L  Q  E  R  S  V  L  V  V  P  R  E  V  T  K  D  P  E  R   255
CTGCTTCGCGTGCTCGAAGATCACCAGATTGAGCGACTGGTGTTGGTACCCTCGCTCCTACGTTCTCTGTTGCTTGCCCTGGGACTCGAA  855
 L  L  R  V  L  E  D  H  Q  I  E  R  L  V  L  V  P  S  L  L  R  S  L  L  L  A  L  G  L  E   285
TCGCGGAACAAGGGCATGTTGAGTCGCCTCAAGACGTGGGTATGCAGTGGAGAACCACTCTCTGCTCAGTTAGCTCGTGAGTTCTTCACT  945
 S  R  N  K  G  M  L  S  R  L  K  T  W  V  C  S  G  E  P  L  S  A  Q  L  A  R  E  F  F  T   315
TATTTTGAGTCAGGCGACCACATCCTCTGTAACTTCTACGGCAGCACTGAAGTGATGGGCGATGTCACATACCACGTGGTGCGCTCCGCT 1035
 Y  F  E  S  G  D  H  I  L  C  N  F  Y  G  S  T  E  V  M  G  D  V  T  Y  H  V  V  R  S  A   345
GCCGAGCTCAAGGACAGCACCAAGGTGCCCATCGGCCGACCCCTGGACAATACCGCCATCTATCTGCTGGACGACAACTTCCGCCCTGTA 1125
 A  E  L  K  D  S  T  K  V  P  I  G  R  P  L  D  N  T  A  I  Y  L  L  D  D  N  F  R  P  V   375
GTTTCAGGTGAAGCGGGAGAACTGTACGTCTCGGGGCTAAACTTGGCACAAGGATATGTGAAGGGTCGGGATCCTGAGAGATTCCTGGCC 1215
 V  S  G  E  A  G  E  L  Y  V  S  G  L  N  L  A  Q  G  Y  V  K  G  R  D  P  E  R  F  L  A   405
AACCCTTTGACCGTGGACCCAGAGCACTCCAGACTGTACCGGACTGGAGACTTCGCACGTTTCGTCAAGGGAACATTGGTGTACGAGGGA 1305
 N  P  L  T  V  D  P  E  H  S  R  L  Y  R  T  G  D  F  A  R  F  V  K  G  T  L  V  Y  E  G   435
CGTACAGATTCACAGGTCAAGATCAGAGGCCACCGTGTGGACTTGGCAGAAGTGGAACGCGCTGTCGCAGGCTTGCCAGGAGTGGACAAA 1395
 R  T  D  S  Q  V  K  I  R  G  H  R  V  D  L  A  E  V  E  R  A  V  A  G  L  P  G  V  D  K   465
TGCGTAGTGCTGTGTTACAAGCCAGGAGAACTTGAGCAGGCACTGCTTGCTTACGTCACAACATGCAAAGATTCATCCATGTCGTCACAG 1485
 C  V  V  L  C  Y  K  P  G  E  L  E  Q  A  L  L  A  Y  V  T  T  C  K  D  S  S  M  S  S  Q   495
CAATTGGAAGCTGTGCTGCACAAGAATCTTGCAGCCTACATGCTACCTCAGGTGTTTGTGGTGGACAGCATCCCATTGCTCGTGAATGGC 1575
 Q  L  E  A  V  L  H  K  N  L  A  A  Y  M  L  P  Q  V  F  V  V  D  S  I  P  L  L  V  N  G   525
AAGACAGACCGACAGGCCTTGCTTCGACGCTATGAGTCATACTCCACCTCCAGAAACAGTGAGTCAGAGGGACCAGAGCTGGACTACACA 1665
 K  T  D  R  Q  A  L  L  R  R  Y  E  S  Y  S  T  S  R  N  S  E  S  E  G  P  E  L  D  Y  T   555
GGGGTGCCAGCCAACCGTCTAGACGCTGCTCGTGTTCTATTTGAGACTGTGGCTGAAGTTCTGGGCGGAGGTACCCGCTCCAAAGTGGGT 1755
 G  V  P  A  N  R  L  D  A  A  R  V  L  F  E  T  V  A  E  V  L  G  G  G  T  R  S  K  V  G   585
CTGCAAGCGAACTTTTATGAACTGGGAGGCAACTCACTTAATTCCGTATTCACCGTGACCAAACTGAGGCAGCTGGGCTACACTATCGGC 1845
 L  Q  A  N  F  Y  E  L  G  G  N  S  L  N  S  V  F  T  V  T  K  L  R  Q  L  G  Y  T  I  G   615
ATCACGGACTTCATCTCGTCTGTGAACTTGCAGCAAGTCCTGGAGAGGATGCAAAGCAATGACGACACTACAGCAACTGTGAGTGAAAAG 1935
 I  T  D  F  I  S  S  V  N  L  Q  Q  V  L  E  R  M  Q  S  N  D  D  T  T  A  T  V  S  E  K   645
CGGGAGAGTAAGAGATACAGTGCAGAGATGCTCCAGGACCAGCATAAACAGTCTGTGAACCACATGATCACGGAGAGCTTTTATGAGAAG 2025
 R  E  S  K  R  Y  S  A  E  M  L  Q  D  Q  H  K  Q  S  V  N  H  M  I  T  E  S  F  Y  E  K   675
GCAGACTTGGAACAGTGGCTCAAGCCAGATATACATCGCGAGGACTACAAGGACCTTACTGACAAGCTCTGGGAACCTCTCGTTCAGAAA 2115
 A  D  L  E  Q  W  L  K  P  D  I  H  R  E  D  Y  K  D  L  T  D  K  L  W  E  P  L  V  Q  K   705
AACCTCAGCTTCATTGTTAAGGACACGCAGGGCGTGCCTGTGGGGGTTGCACTGAACTTCGATGCACATGATGAGCCTGCAGTAGAGATC 2205
 N  L  S  F  I  V  K  D  T  Q  G  V  P  V  G  V  A  L  N  F  D  A  H  D  E  P  A  V  E  I   735
ACAAGCAAGCTGCTTATTGTGTTCGATTTCCTGGAGTTCCTGGAGGGTCCCATAAGGGACAACAAGTTGCCCCAAGGCAAGGGTCAGGTT 2295
 T  S  K  L  L  I  V  F  D  F  L  E  F  L  E  G  P  I  R  D  N  K  L  P  Q  G  K  G  Q  V   765
CTACACAGCTTCATGATGGCCACCCACCCTTCACTGAACTATCAGCAAAACGTCGAGGTCATTCAGTTCATGGAAGCTGAAGTGCTGACG 2385
 L  H  S  F  M  M  A  T  H  P  S  L  N  Y  Q  Q  N  V  E  V  I  Q  F  M  E  A  E  V  L  T   795
ATAGCACACAGCAGGGGCTTTGCAGGCATCTTCACTACAAACACCAATCCCCTAACACAGCAACTGGGAACTGATATATACGACTACAAG 2475
 I  A  H  S  R  G  F  A  G  I  F  T  T  N  T  N  P  L  T  Q  Q  L  G  T  D  I  Y  D  Y  K   825
GTATTCAATGACTACCAGGTGAATCTGTATGAGGCCCCAGATGGATCCAAGCCCTTCAGTGAGGCACCCGACAGCCAAAGAGCAGTCTGC 2565
 V  F  N  D  Y  Q  V  N  L  Y  E  A  P  D  G  S  K  P  F  S  E  A  P  D  S  Q  R  A  V  C   855
TCCTGGAGACCCATTTAAGTACGTTCACCCAGATGCAGATGATCTAGTCTAAGAGGAAGGAGCAGTTGTGGCAACTTACTAAACAACACC 2655
 S  W  R  P  I                                                                              860
TTTGTATCCTATTATAATAATAGTGTTAATTTTAGTTTTTAAGTTCAATCCTAAGTATTTATATATTTTGTACATATTTAAGCTATTGTA 2745
TACAGTATGTGTCATGGTCAATAAAATGTAAAAACCTGTCAAAAAAAA                                            2793
```

Figure 1.

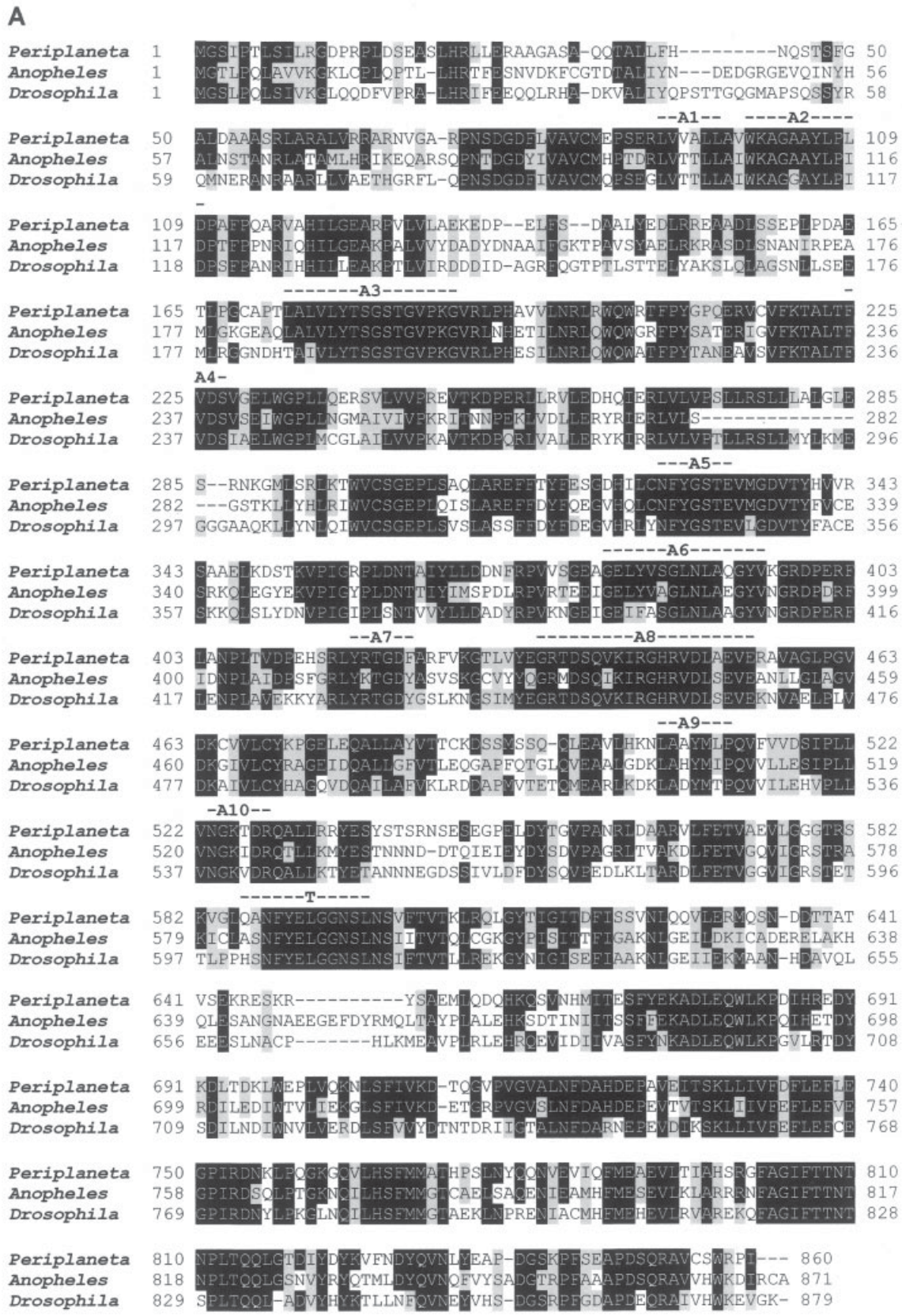
A
Periplaneta
Anopheles
Drosophila
--A1-- ----A2----
-------A3-------
A4-
---A5--
------A6-------
--A7--
--------A8---------
--A9---
-A10--
------T------

Figure 2.

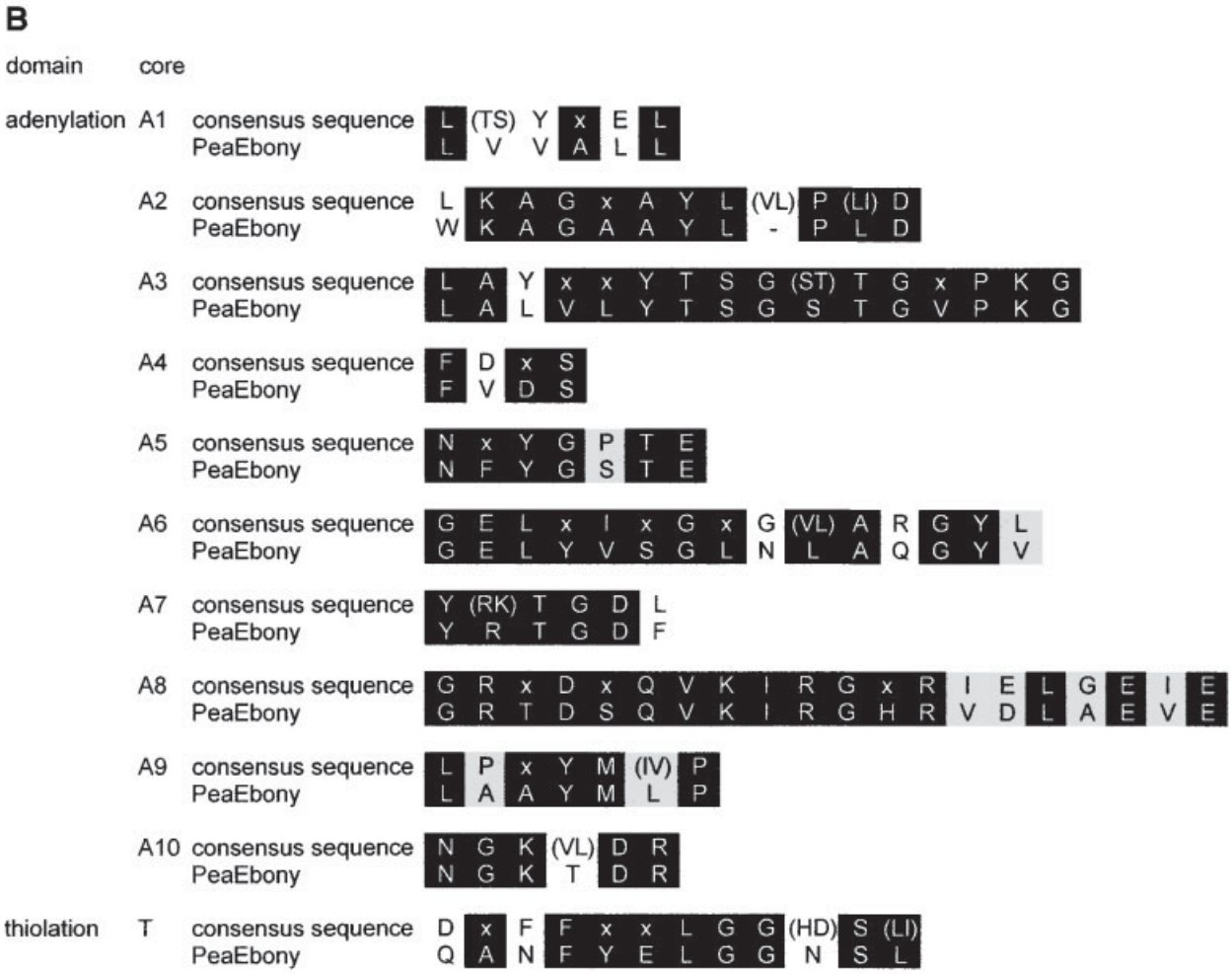

Fig. 2. Alignment of the amino acid sequences of insect Ebony proteins and NRPS sequences. **A:** An alignment of the deduced amino acid sequences of Ebony proteins from *Periplaneta americana*, *Anopheles gambiae* (acc. no. XP_307887), and *Drosophila melanogaster* (acc. no. CAA11962) is shown. The amino acid positions are indicated at the beginning and the end of each line. Identical residues to PeaEbony are depicted as white letters on black; conservative substitutions are shaded. Dashes indicate gaps that were introduced to maximize homologies. Signature sequences for adenylation (A1 to A10) and thioester formation (T) are indicated (Marahiel et al., 1997, Richardt et al., 2003). **B:** Sequence comparison of signature motives for adenylation and thioester formation of NRPSs and PeaEbony. Identical and conservatively substituted residues are labeled as in A.

PeaEbony shows a high degree of similarity to Ebony proteins of other insect species (Fig. 2A). The greatest similarity (identical and conservatively substituted amino acids) of 70.8% exists between PeaEbony and the protein ENSANGP00000021728 of *Anopheles gambiae* (accession no. (#) XP_307887). PeaEbony is also closely related, with ~69% similarity, to the Ebony protein of *Drosophila* (#CAA11962), whereas sequence conservation seems to be much less pronounced (~56%) to the Ebony protein predicted from the honeybee genomic database (#XP_392634). This smaller similarity value, however, might be explained because the honeybee protein is N-terminally incomplete. As described for *Drosophila* Ebony (Hovemann et al., 1998; Richardt et al., 2003), PeaEbony also shares homology to a family of NRPSs and to enzymes involved in siderophore, depsipeptide, and

polyketide biosynthesis, and to adenylate-forming enzymes such as luciferase or acetyl-CoA synthetase (Stachelhaus and Marahiel, 1995; Marahiel et al., 1997; Gehring et al., 1997; Hovemann et al., 1998). These enzymes possess a modular structure with common core sequences in each module (Marahiel et al., 1997; Richardt et al. 2003). Sequence similarity between PeaEbony and NRPSs is striking for the consensus sequence motives A1 to A10 of the adenylation domain and T of the thiolation domain (Fig. 2B). An invariant Ser residue in the thiolation domain of NRPSs, which is important for binding the cofactor 4-phosphopantetheinyl, is conserved in PeaEbony (S_{597}) and *Drosophila* ebony (S_{611}; Richardt et al., 2003). Interestingly, the C-terminal 230 amino acid residues of PeaEbony do not share homologies with NRPSs.

A multiple sequence alignment of four insect Ebony proteins and 16 microbial enzymes was performed and used to build a phylogenetic tree (Fig. 3). This tree showed that PeaEbony clustered with Ebony sequences of *Anopheles*, *Apis*, and *Drosophila* in a distinct clade, a result that agreed well with our BLAST search results.

The tissue-specific expression pattern of Pea*ebony* was determined by RT-PCR. Specific fragments were amplified on RNA samples prepared from brains and salivary glands (Fig. 4, lanes 3 and 4). Small amounts of PCR products were detected in samples prepared from Malpighian tubules (Fig. 4, lane 5), midgut (Fig. 4, lane 6), and muscle (Fig. 4, lane 7). These amplification products are unlikely to have arisen from genomic DNA because controls treated with an RNase cocktail did not result in the amplification of PCR products (Fig. 4, lanes 8 to 12).

DISCUSSION

In the present study, we have identified and characterized ebony cDNA from the American cockroach, *Periplaneta americana*. BLAST searches have revealed homologies not only to Ebony proteins of other insect species but also to a family of NRPSs expressed in bacteria and filamentous fungi. Sequence similarity has also been observed between PeaEbony and enzymes involved in siderophore, depsipeptide, and polyketide biosynthesis and adenylate- forming enzymes such as luciferase or acetyl-CoA synthetase (Stachelhaus and Marahiel, 1995; Marahiel et al., 1997; Gehring et al., 1997; Hovemann et al., 1998). All these enzymes have one property in common: the ATP-dependent carboxy acid activation by acyladenylate. Except for luciferase and acetyl-CoA synthetase, the functional relatedness also extends to a thiolation module. Here, a 4′-phosphopantetheinyl cofactor binds and converts the apo to the holo enzyme (Lambalot et al., 1996). The adenylation domain (A) in all these proteins spans about 550 amino acids. It is directly involved in substrate binding and activation by ATP (Marahiel et al., 1997). In type I peptide synthetases, this domain is directly followed by the thiolation domain (T) spanning about 100 amino acids. The pronounced conservation of sequence motives A1 to A10 and T in PeaEbony and in peptide synthetases type I assigns PeaEbony as a member of this peptide synthetase family. Like *Drosophila* Ebony, PeaEbony most likely acts as a β-alanyl-biogenic amine synthetase. The striking conservation of the type I peptide synthetase core sequences in Ebony proteins (Fig. 2a; Richardt et al., 2003) has encouraged Hovemann and co-workers to propose and experimentally establish (Hovemann et al., 1998; Richardt et al., 2003) a model for the reaction mechanism. In the first step, β-alanine bound to the adenylation domain is activated by ATP, forming β-alanyl-adenylate. In the second step, the β-alanyl-adenylate is intramolecularly transferred to a 4′-phosphopantetheinyl group bound to a serine residue within the thiolation domain. A thioester is formed, and AMP is released. The β-alanine then reacts with the primary amino group of a biogenic amine (e.g., dopamine) yielding the β-alanyl conjugate of the biogenic amine (e.g., β-alanyl dopamine). According to this mechanism, Ebony enzymes must have the capacity to bind biogenic amines to allow peptide bond formation. Substrate (= biogenic amine) binding most likely occurs within the C-terminal 230 amino acid residues that do not share significant homologies with

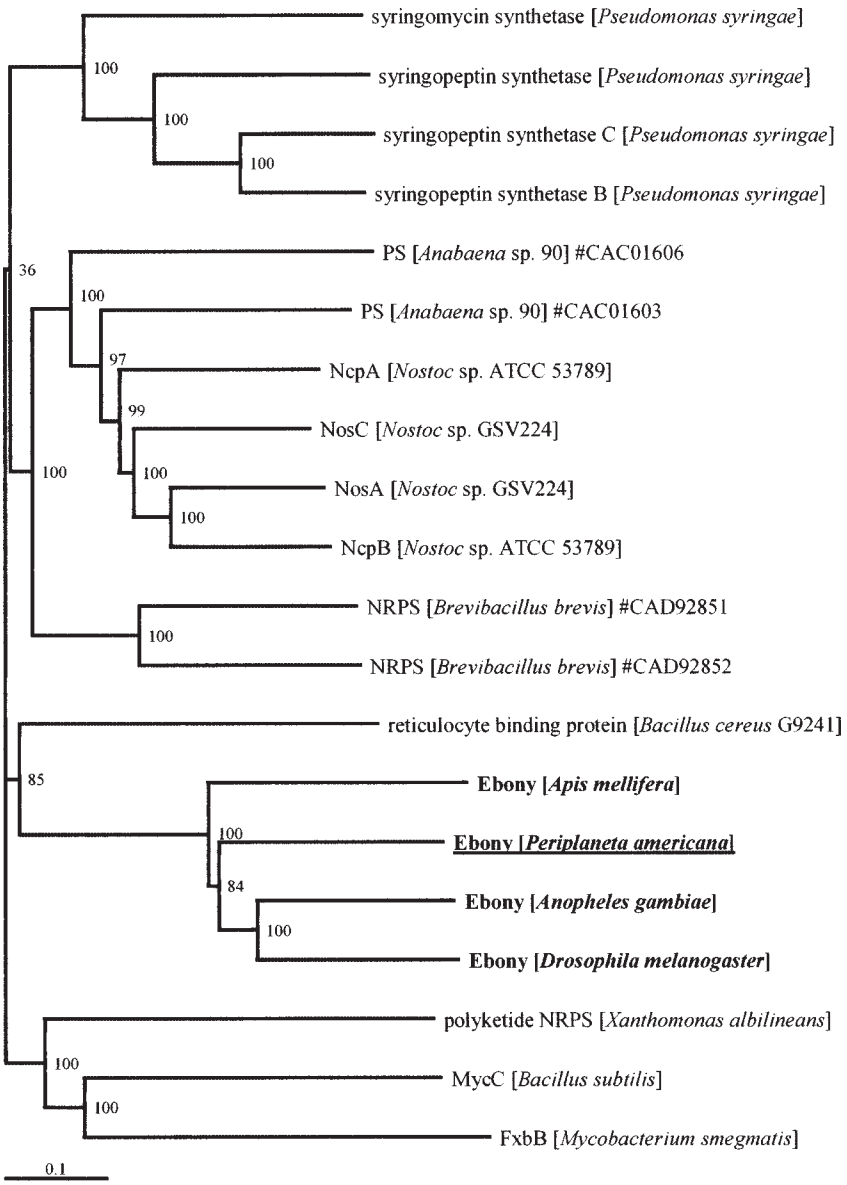

Fig. 3. Phylogram of insect Ebony proteins and microbial peptide synthetases. The multiple alignment was performed with the complete amino acid sequence of each protein. The genetic distance was calculated with ClustalX (version 1.81). The protein sequences, followed by their GenBank accession numbers (#), are listed in the order illustrated: syringomycin synthetase [*Pseudomonas syringae*] (#AAC80285), syringopeptin synthetase [*Pseudomonas syringae*] (#AAF99707), syringopeptin synthetase C [*Pseudomonas syringae*] (#AAO72425), syringopeptin synthetase B [*Pseudomonas syringae*] (#AAO72424), peptide synthetase [*Anabaena* sp. 90] (#CAC01606), peptide synthetase [*Anabaena* sp. 90] (#CAC01603), NcpA [*Nostoc* sp. ATCC 53789] (#AAO23333), NosC [*Nostoc* sp. GSV224] (#AAF17280), NosA [*Nostoc* sp. GSV224] (#AAF15891), NcpB [*Nostoc* sp. ATCC 53789] (#AAO23334), nonribosomal peptide synthetase [*Brevibacillus brevis*] (#CAD92851), nonribosomal peptide synthetase [*Brevibacillus brevis*] (#CAD92852), reticulocyte binding protein [*Bacillus cereus* G9241] (#ZP_00236768), similar to ENSANGP00000021728 [*Apis mellifera*] (#XP_392634), Ebony [*Periplaneta americana*] (#AJ865468), ENSANGP00000021728 [*Anopheles gambiae*] (#XP_307887), Ebony [*Drosophila melanogaster*] (#CAA11962), Polyketide non-ribosomal peptide synthetase [*Xanthomonas albilineans*] (#CAE52339), MycC [*Bacillus subtilis*] (#AAF08797), and FxbB [*Mycobacterium smegmatis*] (#AAC32047).

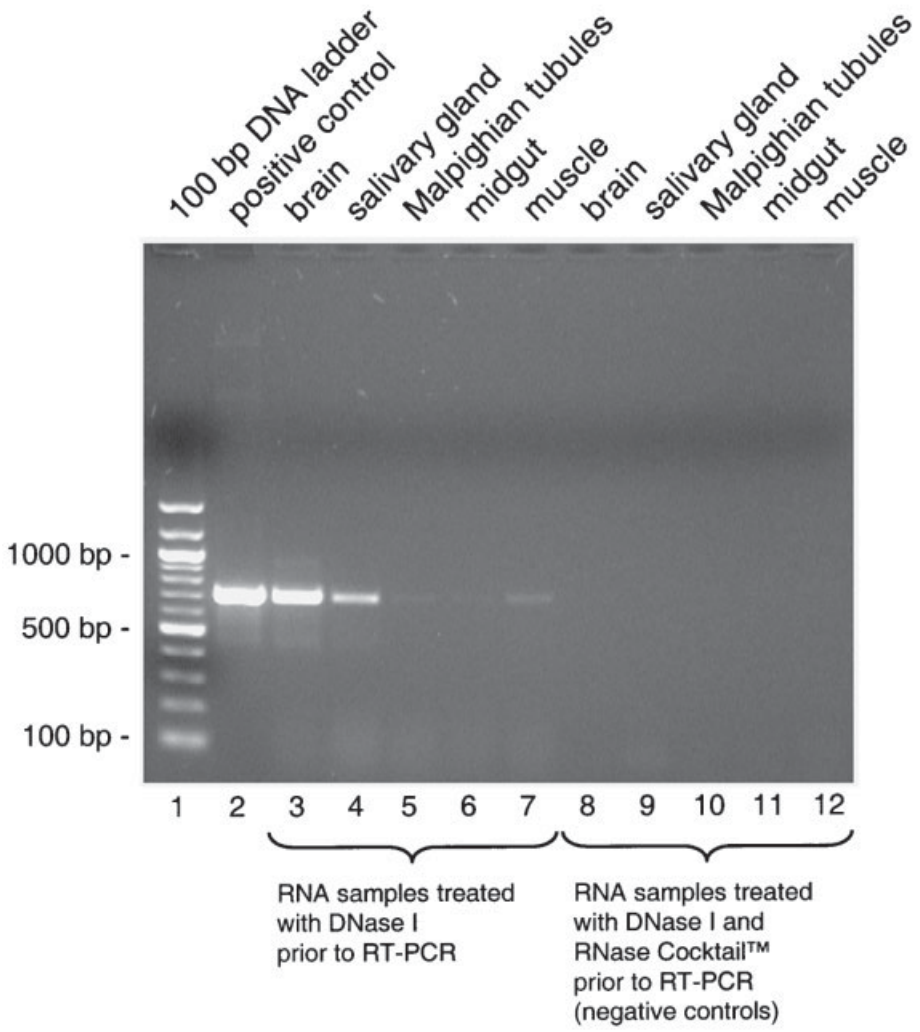

Fig. 4. Expression of the *ebony* gene in various tissues of *Periplaneta americana*. **Lane 1:** The 100-bp DNA ladder (New England BioLabs, Germany). **Lane 2:** PCR-amplification performed on a pBluescript clone harboring the Pea*ebony* cDNA served as a positive control. Consecutive lanes contain RT-PCR products amplified from brain RNA (**lane 3**), salivary gland RNA (**lane 4**), Malpighian tubule RNA (**lane 5**), midgut RNA (**lane 6**), and muscle-specific RNA (**lane 7**). Amplified fragments have the expected size of 707 bp. Amplification, however, failed on RNA samples that were pre-digested with an RNase cocktail prior to RT-PCR (**lanes 8 to 12**).

NRPSs (Hovemann et al., 1998; Richardt et al., 2003).

Pea*ebony* mRNA is highly expressed in the brain of *Periplaneta americana*. This is in good agreement with the high expression level of *Drosophila ebony* in the optic lobes of the fly brain (Hovemann et al., 1998; Richardt et al., 2002). Recently, Pérez et al. (2004) reported constitutive activity of an "*N*-β-alanyl-catecholamine ligase" in the brain of the medfly *Ceratitis capitata* and other insect species including the cockroach *Blatella germanica*. *Ceratitis capitata* brain extracts were found to synthesize β-alanyl conjugates not only from dopamine and norepinephrine, but also from tyramine, octopamine, serotonin, and histamine (Pérez et al., 2004). It is tempting to speculate that this "*N*-β-alanyl-catecholamine ligase" activity in *Ceratitis capitata* is associated with Ebony protein expression. Pea*ebony* mRNA is also expressed in the salivary glands of the cockroach. The acinar salivary glands of

Periplaneta americana are richly innervated by dopaminergic and serotonergic neurons (Baumann et al., 2002, 2004). The stimulation of isolated glands with dopamine induces the production of protein-free saliva, which is secreted exclusively by the acinar peripheral cells (Just and Walz, 1996; Marg et al., 2004). On the other hand, serotonin stimulates acinar peripheral and central cells (Just and Walz, 1996). The central cells secrete proteins into the acinar lumen resulting in the production of protein-containing saliva (Just and Walz, 1996). Although we have not yet examined Pea*ebony* expression at the cellular level either in the brain or in the salivary gland complex, the enzyme in general is well suited to inactivate biogenic amines and thereby to terminate aminergic signal transduction in the brain and in the salivary gland.

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