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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*)-flupentixo 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 significantl increased levels of cAMP even in the absence of ligand. This constitutive activity was blocked by *cis*(*Z*)-flupentixol This work provides the firs 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.
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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 specifi 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 profile (reviewed in Refs. [30,40,48]). For example, the benzazepine, *R*(+)-SCH 23390, binds with much higher affinit to vertebrate D1 receptors than to D2 receptors, whereas benzamides, such as sulpiride and eticlopride, and the butyrophenone, spiperone, show higher affinit 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 significantl 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 profil typical of a vertebrate D1 receptor. For example, the vertebrate D1 receptor antagonist, SCH 23390, does not exhibit high affinit 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 profil that is distinct from that of vertebrate D1 receptors [3]. Most strikingly, the vertebrate D2 receptor antagonist spiperone was found to have a higher affinit 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

21. 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 purifie 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.

22. 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.

23. 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 fixe 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 purifie 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 fil for 12–72 h at −80 °C. Quantitation of Northern blots and ethidium bromide stained gels was done using NIH Image.

24. 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 figur 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.

25. Pharmacology of the AmDOP2 receptor

A pharmacological profil 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 profil 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.

26. *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 figur legends.

27. *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, fluphenazin dihydrochloride, domperidone, *cis*(*Z*)-flupen tixol 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.

28. *Data analysis*

Data were analyzed and displayed using Prism 3.0a for Macintosh (GraphPad Software, San Diego, CA, USA, www.graphpad.com). Curve fittin was done using least-squares analysis. Statistical significanc 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

31. *Analysis of the activity of biogenic amines at the AmDOP2 receptor*

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

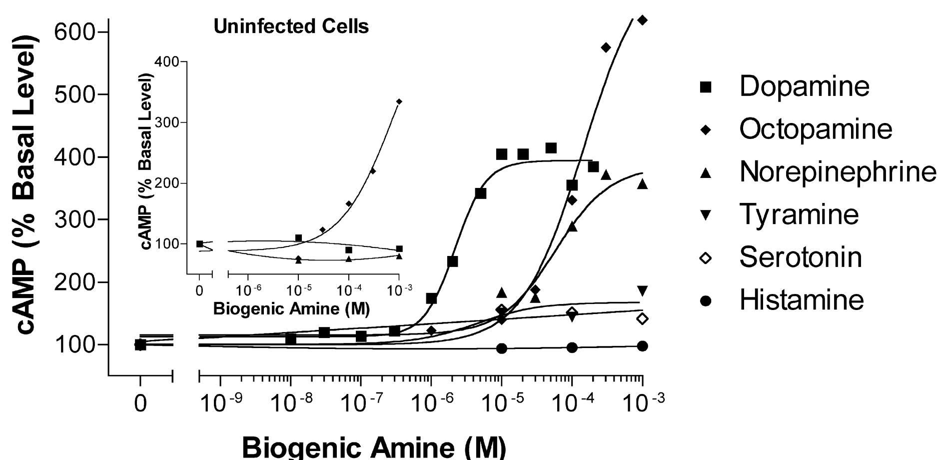

Fig. 1. Biogenic amine-mediated changes in intracellular cAMP level. Responses were examined in Sf21 cells expressing the *Am*DOP2 receptor, as well as in control (uninfected) cells (inset). All measurements are expressed relative to basal (cAMP detected in the absence of ligand), which provides the 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.

32. Identificatio 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 significantl 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 significantl different from basal levels recorded in the absence of dopamine (Fig. 2B). Significantl 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.

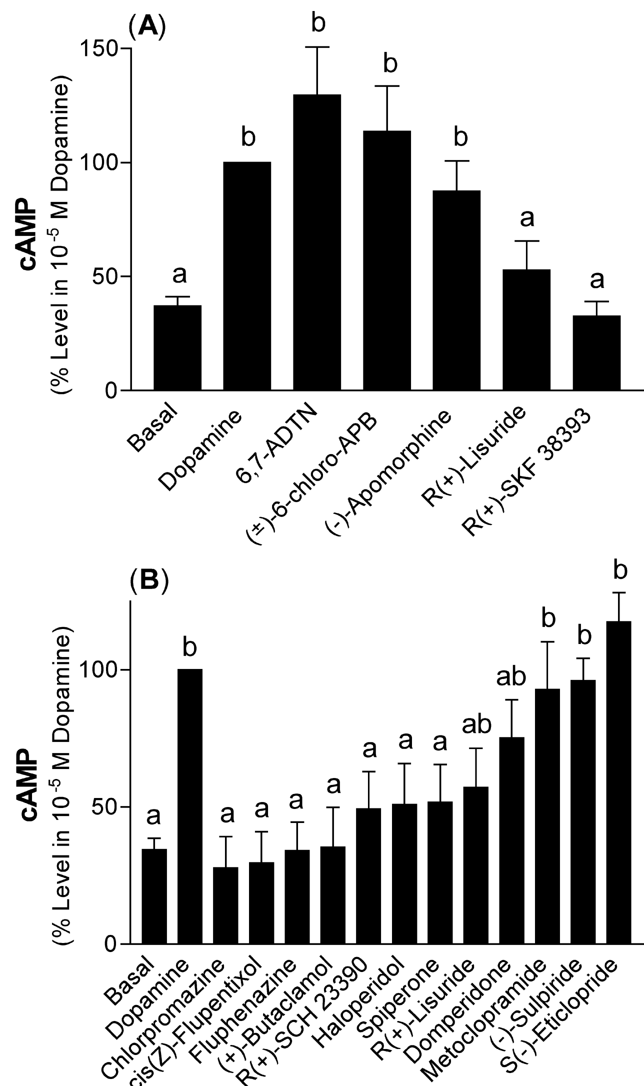

Fig. 2. Responses of Sf21 cells expressing the *Am*DOP2 receptor to (A) selected dopamine receptor agonists at a concentration of 10^{-5} M, and (B) dopamine (10^{-5} M) plus 10^{-5} M of one of a selection of amine receptor antagonists. Values are expressed as a percentage of the cAMP level in 10^{-5} 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 significanc 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 significantl different ($P>0.05$).

33. 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 specificit 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.

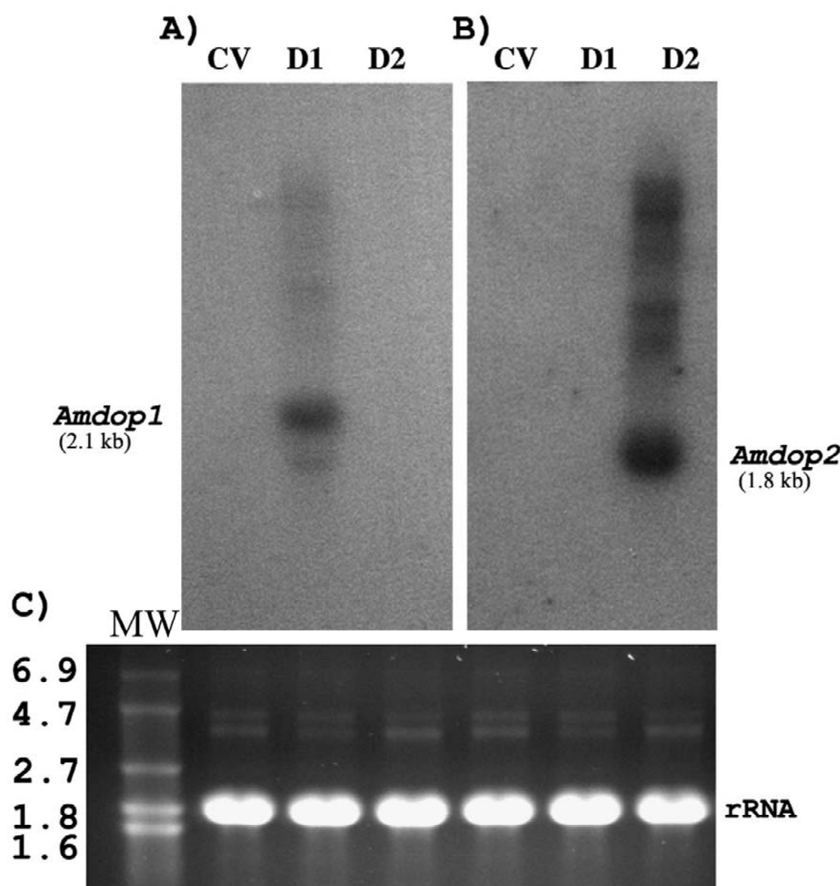

Fig. 3. Northern analysis of *Amdop2* and *Amdop1* expression levels. Total RNA was isolated from Sf21 cells infected with baculovirus expressing *Amdop2* (D2), *Amdop1* (D1), or no receptor (control virus, CV). (A) Membrane probed with *Amdop1* shows a specifi band at 2.1 kb, the expected size for the *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, specifi 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 flupentixo to levels not

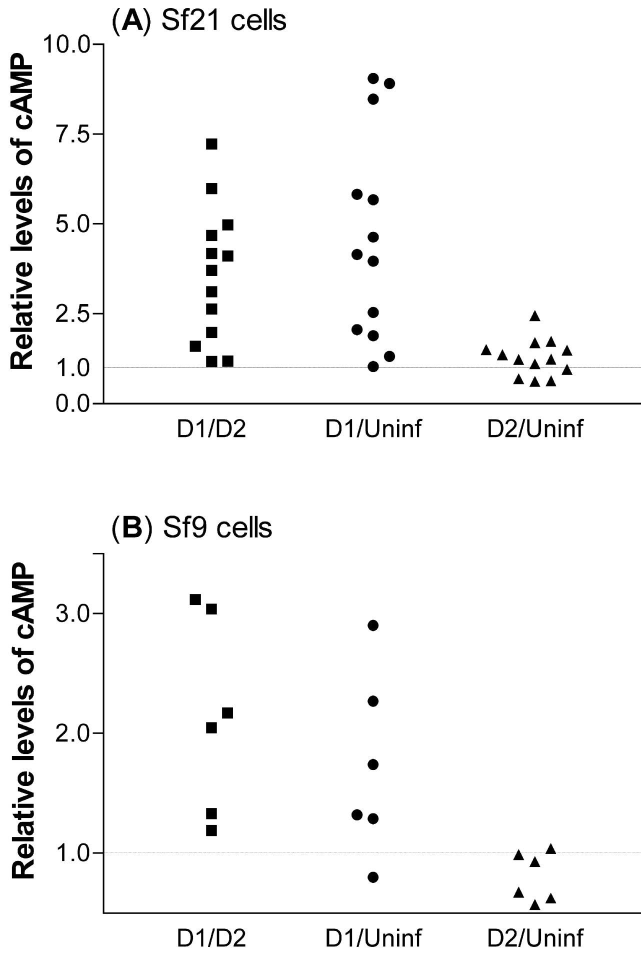

Fig. 4. Relative levels of intracellular cAMP in cells expressing *Am*DOP1 receptors, *Am*DOP2 receptors, or neither receptor (uninfected cells). Basal levels of cAMP (measured in 100 μM IBMX) determined for cells expressing *Am*DOP1 receptors (D1), *Am*DOP2 receptors (D2) and uninfected cells (Uninf) 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 significantl 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.)

significantl 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 flupentixo 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 flupentixo concentrations revealing that flupentixo 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'.

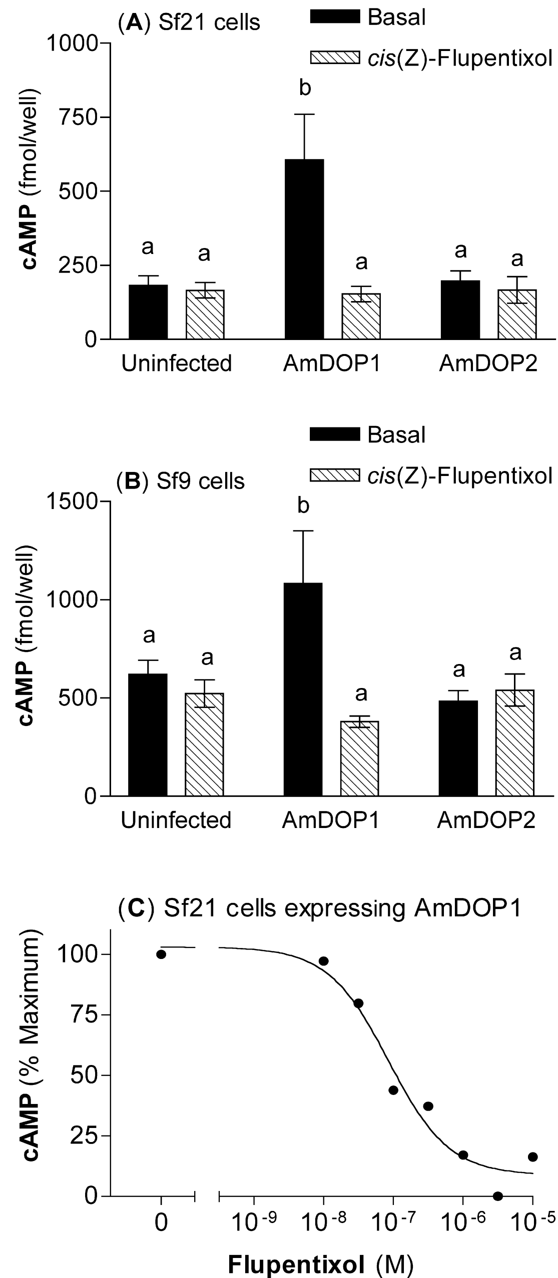

Fig. 5. Effect of flupentixo on basal cAMP levels in cells expressing the *Am*DOP2 receptor, the *Am*DOP1 receptor, or neither receptor (uninfected cells). (A) Sf21 cells or (B) Sf9 cells were treated with either, IBMX alone (Basal), or with IBMX plus 10^{-5} M *cis*(*Z*)-flupentixol Values shown are means±S.E.M. for six independent experiments. Overall statistical significanc 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 significantl 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 flupentixo 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.

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-

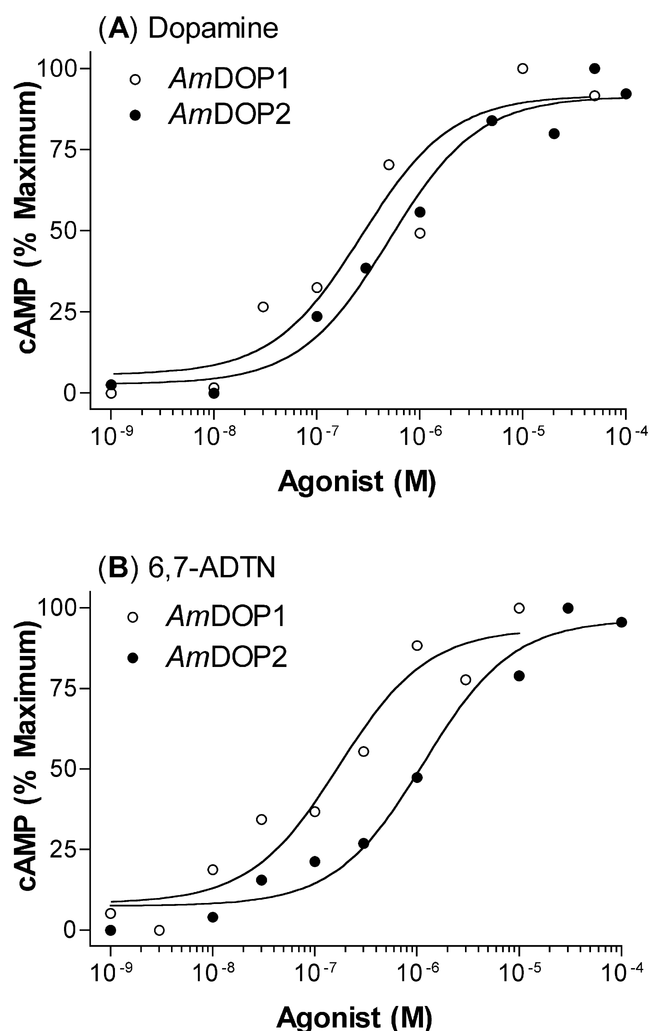

Fig. 6. Agonist response curves for Sf21 cells expressing either *Am*DOP1 or *Am*DOP2 receptors. For these experiments, cells expressing *Am*DOP2 receptors were examined in parallel with cells expressing *Am*DOP1 receptors. To allow comparison, the data have been normalized with the minimum cAMP level 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.

mediated stimulation of cAMP in cells expressing *Am*DOP1 (Fig. 7A) or *Am*DOP2 (Fig. 7B) revealed that at both receptors, flupentixo was the most potent of the antagonists tested, followed by butaclamol, spiperone and then SCH 23390 (Table 1).

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

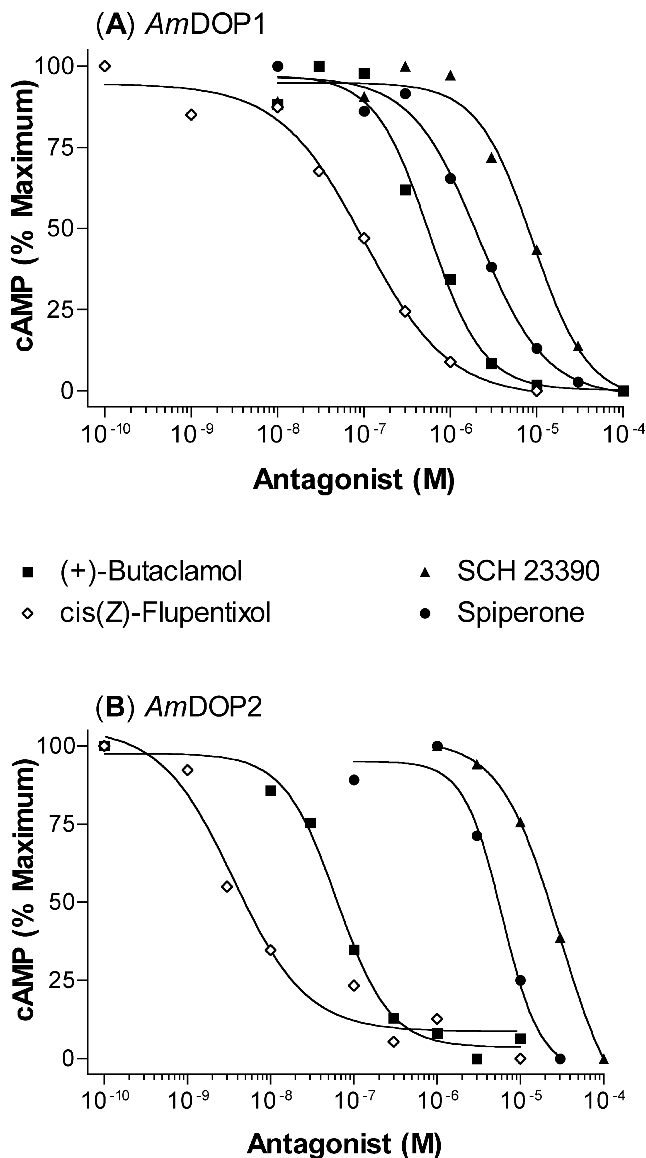

Fig. 7. Effects of dopamine receptor antagonists acting via *Am*DOP1 receptors (A) or *Am*DOP2 receptors (B). (A) Cells expressing *Am*DOP1 receptors were exposed to dopamine (10^{-6} M) and the selected antagonist at the range of concentrations indicated. (B) Cells expressing *Am*DOP2 receptors were 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 firs 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 significan 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 flupentixo (see Fig. 5), in reducing the agonist-independent activity of constitutively activated receptors is also of great interest, and may be highly significan in terms of the functional properties of such receptors. While endogenous inverse agonists have yet to be identifie 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 identifie 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 affinit for dopamine than the D1/D1A receptor. The antagonists flupentixo and butaclamol, on the other hand, have a higher affinit 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 firs 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 significan 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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