

Arthropod D₂ receptors positively couple with cAMP through the Gi/o protein family

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Abstract

The pyloric network is an important model system for understanding neuromodulation of rhythmic motor behaviors like breathing or walking. Dopamine (DA) differentially modulates neurons within the pyloric network. However, while the electrophysiological actions of DA have been well characterized, nothing is known about the signaling events that mediate its effects. We have begun a molecular characterization of DA receptors (DARs) in this invertebrate system. Here, we describe the cloning and characterization of the lobster D₂ receptor, D_{2αPan}. We found that when expressed in HEK cells, the D_{2αPan} receptor is activated by DA, but not other monoamines endogenous to the lobster nervous system. This receptor positively couples with cAMP through multiple Gi/o proteins via two discrete pathways: 1) a G_α mediated inhibition of adenylyl cyclase (AC), leading to a decrease in cAMP and 2) a G_{βγ}-mediated activation of phospholipase C_β (PLC_β), leading to an increase in cAMP. Alternate splicing alters the potency and efficacy of the receptor, but does not affect monoamine specificity. Finally, we show that arthropod D₂ receptor coupling with cAMP varies with the cellular milieu.

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1. Introduction

The crustacean stomatogastric ganglion is extensively used as a model to understand neuromodulatory effects on motor pattern generation (Nusbaum and Beenhakker, 2002; Harris-Warrick and Marder, 1992). A wealth of information exists on the monoaminergic modulation of ion currents and neuronal firing properties (Flamm and Harris-Warrick, 1986; Harris-Warrick et al., 1995b,a, 1998; Kloppenburg et al., 1999; Johnson et al., 2003), but nothing is known about the transduction cascades mediating these effects. To extend the usefulness of this model system and gain insight into how

component neurons integrate biochemical and electrical processes, we have begun a molecular characterization of DARs in this central pattern generator (CPG).

In the traditional view, DARs are classified as type-1 or type-2: type-1 DARs couple to G_s proteins, leading to a G_α mediated increase in [cAMP]_i and protein kinase A (PKA) activity, while type-2 DARs couple to Gi/o proteins to decrease [cAMP]_i and PKA activity (Missale et al., 1998; Neve et al., 2004). It is now clear that this traditional view of DAR signaling is much too simple. First, DARs have been shown to couple with multiple G proteins in various heterologous and native systems (Kimura et al., 1995a,b; Sidhu et al., 1998; Zheng et al., 2003; O'Sullivan et al., 2004; Zhen et al., 2004). Moreover, GPCRs, including DARs, can switch G protein coupling over time in response to constant agonist application (Daaka et al., 1997; Baillie et al., 2003; Lezcano et al., 2000). Second, both the G_α and G_{βγ} subunits are known to mediate individual responses (Cabrera-Vera et al., 2003). Third, activated G protein subunits can directly interact with target proteins such as ion channels without altering second messenger levels (Dascal, 2001; Ivanina et al., 2004). Fourth, GPCRs are known to interact

Abbreviations: CPG, central pattern generator; GPCR, G protein coupled receptor; DA, dopamine; DAR, dopamine receptor; PKA, protein kinase A; PP2A, protein phosphatase 2A; PTX, pertussis toxin; Et-18-OCH₃, 1-*O*-Octadecyl-2-*O*-methyl-rac-glycero-3-phosphorylcholine; IBMX, 3-isobutyl-1-methylxanthine; FSK, forskolin; 5-HT, serotonin; OCT, octopamine; TYR, tyramine; HIS, histamine; AC, adenylyl cyclase; PLC, phospholipase C.

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directly with target proteins. For example, DARs can physically interact with, and activate ionotropic glutamate receptors (Zou et al., 2005; Lee and Liu, 2004; Pei et al., 2004; Liu et al., 2000). Fifth, GPCRs can activate additional cascades, like the mitogen activated protein kinase (MAPK) cascade via crosstalk (Werry et al., 2005). Finally, GPCRs can directly activate G protein independent cascades. One important mechanism involves recruitment of β -arrestin scaffolds to an activated receptor and subsequent stimulation of G protein independent cascades (Lefkowitz and Shenoy, 2005). In this regard, it was recently shown that the D_2 receptor modulates locomotor activity in mice via a β -arrestin 2-mediated signaling complex involving Akt and PP2A, as well as by traditional G protein cascades (Beaulieu et al., 2005).

There are three arthropod DARs: two type-1 receptors and one type-2 receptor (Gotzes et al., 1994; Feng et al., 1996; Han et al., 1996; Hearn et al., 2002; Blenau et al., 1998; Beggs et al., 2005; Mustard et al., 2003). A fourth arthropod receptor that responds to DA with an increase in cAMP has been cloned, but it is primarily activated by ecdysteroids and does not appear to belong to the DAR family (Srivastava et al., 2005). We have previously cloned and characterized the two type-1 DARs from the spiny lobster (Clark and Baro, 2006). Here we describe the cloning and characterization of the lobster D_2 receptor, $D_{2\alpha}$ Pan.

2. Materials and methods

2.1. Cloning and expression in a heterologous system

The lobster $D_{2\alpha}$ Pan cDNA was cloned from nervous tissue of *Panulirus interruptus* using a degenerate PCR strategy with conventional library screening and RACE technology as previously described (Clark et al., 2004). The $D_{2\alpha}$.1Pan sequence has been submitted to Genbank under accession number DQ900655 (Fig. 1). Full length constructs were created and inserted into a pIRESneo3 vector (B.D. Biosciences Clontech, Palo Alto, CA) using standard recombinant techniques. $D_{2\alpha}$ Pan and *AmDop3* constructs were stably expressed in HEK293 cells using methods previously described (Clark et al., 2004). *AmDop3* was kindly provided by Dr. Allison Mercer, University of Otago. All tissue culture reagents were purchased from Invitrogen except the DMEM and the penicillin streptomycin solution (American Type Culture Collection), and the neomycin (Sigma).

In some experiments, the $G\beta\gamma$ scavengers, dexas1 (UMR cDNA resource center, University of Missouri-Rolla) or β ARK_{495–689} (kindly provided by Dr. Robert Lefkowitz, Howard Hughes Medical Institute), were transiently expressed. In these cases, cells were maintained in DMEM supplemented



Fig. 1. The DAR family is conserved across arthropods. The *Panulirus* (lob), *Drosophila* (fly), *Apis* (bee), and human (hum) DAR proteins are aligned. Amino acids that are identical are highlighted. Black bars approximate the seven transmembrane regions. The points of alternate splicing on lobster DARs are indicated by black arrowheads. The accession numbers are as follows: lobster: DQ900655; fly: AAN15955; bee: NP_001014983; human: NP_000786.

with 10% dialyzed fetal bovine serum plus 600 $\mu\text{g}/\text{mL}$ neomycin (HEKD_{2 α} Pan or HEKAmDop3) or 50 units/mL penicillin and 50 $\mu\text{g}/\text{mL}$ streptomycin (parental HEK cells) at 37 °C, 5% CO₂, and were grown to 90–95% confluency in 26 \times 33 mm wells of an 8-well plate (Fisher Scientific). One day prior to transfection, the cells received media without antibiotic. Cells were transfected with 2 μg DNA using 10 μL lipofectamine in 100 μL opti-MEM according to the manufacturer's instructions. After 6 h at 37 °C, 5% CO₂, cells received 1 mL of DMEM containing 20% dialyzed serum. Cells received normal media (with antibiotic) 24 h following transfection, and were assayed 24–48 h later.

The experiments described in this manuscript were conducted over the course of 2 years, during which time the properties of the parental HEK cell line varied. During the first year the parental line was insensitive to DA, even at a concentration of 100 mM. The assays shown in Figs. 2, 4, 6 and 7 were conducted during this initial period. There was then a long hiatus from experimentation during which time all cell lines were frozen in liquid nitrogen. Experiments were resumed during year 2. Parental HEK and HEKD_{2 α} .1Pan cells were thawed and the assays shown in Figs. 3 and 5 were performed. In addition, the parental line was also transfected to generate stable HEKAmDop3, HEKD_{2 α} .2Pan, and HEKD_{2 α} .3Pan lines, and the assays shown in Figs. 8 and 9 were performed. At some point during the second year the parental line began to express low and variable levels of an endogenous human D₁ receptor that in some assays produced a significant increase in cAMP in response to 10⁻⁴ M DA or the D₁ selective agonist, 6-chloro-PB ($n=3$, $p<0.05$). The pharmacology of the human D₁ receptor was distinct from the D_{2 α} Pan receptor. The D_{2 α} Pan receptor produced an increase in cAMP in response to 10⁻⁵ M quinpirole ($n=3$, $p<0.05$), a selective D₂ agonist, while the parental D₁ receptor did not ($n=3$, $p>0.05$). Furthermore, the signaling properties of the two receptors were distinct: the arthropod D₂ receptor relies on the G $\beta\gamma$ subunit to produce an

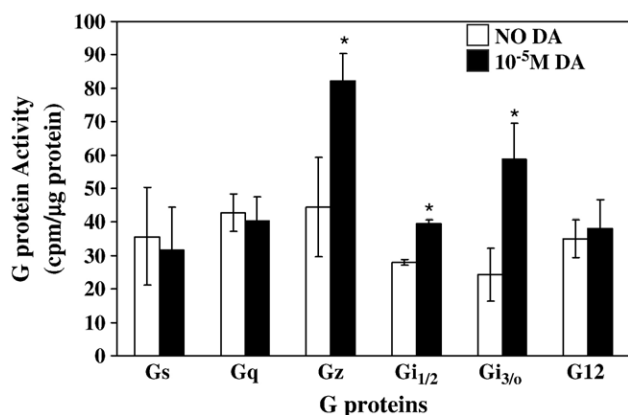


Fig. 2. The D_{2 α} .1Pan receptor couples with Gi/o family members. G protein activities in HEKD_{2 α} .1Pan membrane preparations were measured in the absence (open bar) vs. the presence (filled bar) of 10⁻⁵ M DA for eight G proteins: Gs, Gq, Gz, Gi₁, Gi₂, Gi₃, Go, G12. Data represent the mean \pm S.E.M., $n=3$. Student *t*-tests were performed and statistically significant differences in the activity of a given G protein in the presence vs. absence of DA are indicated with an asterisk ($p<0.05$).

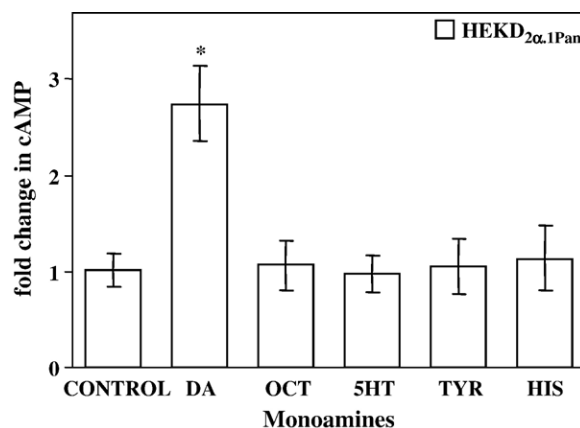


Fig. 3. DA is the only monoamine that activates D_{2 α} .1Pan. Levels of cAMP (pmol cAMP/mg protein) were simultaneously measured in HEK and HEKD_{2 α} .1Pan (open bars) cell lines under control conditions (no monoamines present) or in the presence of 1mM of the indicated monoamine. The cAMP levels measured in the presence of the indicated monoamine were first normalized to control [cAMP] for each cell line and then the HEKD_{2 α} .1Pan response was normalized by the HEK response. Average fold changes in the transfected cell line relative to the parental cell line are plotted, error bars indicate the S.E.M., $n>3$. The data were subjected to a one-way ANOVA. * indicates significant differences from cAMP levels measured in the control ($p<0.05$).

increase in cAMP while the human D₁ receptor does not (Figs. 5 and 8).

2.2. Membrane preparations

Stably transfected cells were harvested with trypsin (ATCC, Manassas, VA). Pellets were homogenized in 20 mM HEPES (pH 7.4) containing 2 mM MgCl₂, 1 mM EDTA, 2 mM 1,4-dithiothreitol (DTT), 1 $\mu\text{g}/\text{mL}$ leupeptin, 1 $\mu\text{g}/\text{mL}$ aprotinin, and 2 mM PMSF. The homogenate was centrifuged at 2500 rpm for 5 min. The supernatant was recovered and centrifuged at 15,000 rpm for 30 min at 4 °C. Pellets were resuspended in 20 mM HEPES (pH 7.4) containing 0.5% 3-[(3 cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and 2 mM EDTA. Protein concentrations in each sample were determined using a BCA Protein Assay Kit (Pierce).

2.3. G protein activation assay

Agonist-induced activation of specific G proteins was determined using our previously described G protein activation assay (Clark and Baro, 2006). Briefly, membrane preparations from cell lines (1.5 $\mu\text{g}/\mu\text{L}$ of protein) were incubated at 37 °C for 15 min in 10 mM HEPES (pH 7.4) containing 10 mM MgCl₂, 100 μM EDTA and 10 nM GTP γ ³⁵S (Amersham) with or without DA. Reactions were terminated with ten volumes of termination buffer [10 mM MgCl₂, 100 μM GDP, 200 mM NaCl in 100 mM Tris (pH 8.0)]. Fifty μL of each terminated sample were then aliquotted in triplicate to wells precoated with one antibody against a human G α subunit [G_{12 α} , G_{11/2 α} , G_{q α} , G_{13/0 α} , G_{s α} , or G_{z α} (EMD/Calbiochem)] and to uncoated wells (blanks). Plates were incubated on ice for 2 h. Wells were then rinsed three times with phosphate-buffered saline containing 0.3% Tween-20. Individual wells were placed in scintillation

vials containing ScintiSafe Econo 1 (Fisher) and the radioactivity in each well was quantified with a scintillation counter. Resulting cpm from the blank wells were averaged and used as a measure of non-specific binding. The non-specific binding was subtracted from the average cpm obtained from the coated wells. Data are expressed as cpm/ μg of protein.

2.4. cAMP assays

cAMP levels were measured as previously described (Clark et al., 2004). Briefly, 1×10^5 cells were plated in 35 mm dishes and grown to confluence. Cells were washed with 1 mL of media and preincubated at 37 °C for 10 min in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (2.5 mM) (Sigma). Cells were incubated an additional 30 min at 37 °C with or without forskolin (2.5 μM), and varying concentrations of monoamine (DA, 5-HT, tyramine, histamine, or octopamine). In some experiments, cells were pretreated for 24 h with pertussis toxin (PTX, Calbiochem) or 15 min with 1-*O*-Octadecyl-2-*O*-methyl-rac-glycero-3-phosphorylcholine (Et-18-OCH₃, Calbiochem). The media was removed and 0.5 mL of 0.1 M HCl with 0.8% Triton X-100 (Sigma) was added to the plates. After a 30 min incubation at room temperature, the lysate was removed from the plates and centrifuged for 2 min. The supernatant was collected and assayed for cAMP levels using a direct cAMP enzyme immunoassay kit (Assay Designs, Inc.) according to the manufacturer's instructions. Protein concentrations in each sample were determined using a BCA Protein Assay Kit (Pierce).

2.5. Statistical analyses and curve fitting

Student *t*-tests were performed with Excel software. Curve fitting, Kruskal–Wallis (ANOVA on ranks) tests, and Bonferroni posttests were performed with Prism (GraphPad Software, San Diego, CA, www.graphpad.com). In all cases, statistical significance was determined as $p < 0.05$.

3. Results

3.1. DARs are conserved across species

Using total lobster nervous system cDNA and a combination of conventional library screening and RACE technologies, we

cloned a type-2 DAR from the spiny lobster, *P. interruptus*. We found that this receptor, $D_{2\alpha\text{Pan}}$, is alternately spliced (see arrowheads in Fig. 1) to produce four distinct proteins: $D_{2\alpha.1\text{Pan}}$, $D_{2\alpha.2\text{Pan}}$, $D_{2\alpha.3\text{Pan}}$, and $D_{2\alpha.4\text{Pan}}$ (Table 1). We did not conduct an exhaustive search for $D_{2\alpha\text{Pan}}$ isoforms, and it is likely that additional splice forms exist (Hearn et al., 2002).

The $D_{2\alpha\text{Pan}}$ receptor is orthologous to the *Drosophila* receptor DD2R (Hearn et al., 2002) and the *Apis* receptor Am-Dop3 (Beggs et al., 2005). A BLAST against the *Homo sapiens* Reference Proteins database showed that the $D_{2\alpha\text{Pan}}$ receptor was most homologous to the long form of the human D_2 receptor (NP_000786) with an *E* value of 3e-58. Fig. 1 illustrates that all D_2 receptors are well conserved across species. When compared to its fly, honeybee and human homologs, the $D_{2\alpha\text{Pan}}$ receptor shows 45%, 39% and 37% amino acid identity over the entire protein, respectively. As expected, the 7 transmembrane regions are among the most conserved portions of the protein. In addition, the cytoplasmic domains known to interact with G proteins show a fairly high degree of identity, including intracellular loops 1 and 2, the amino and carboxy portions of intracellular loop 3, and the cytoplasmic C-terminal domain (Limbird, 2004; Cabrera-Vera et al., 2003). Most amino acid substitutions in these regions are conservative (Fig. 1).

3.2. $D_{2\alpha.1\text{Pan}}$ couples with multiple members of the Gi/o family in HEK cells

As stated above, the G protein interaction domains are well conserved between arthropodal and mammalian D_2 receptors. Similarly, the G protein domains that interact with receptors are conserved across species (reviewed in Cabrera-Vera et al., 2003). The last five residues of the $G\alpha$ C-terminus is an important mediator of receptor-G protein interactions. This domain shows 100% amino acid identity between human and arthropod Gs, Gi, Go and Gq homologs (reviewed in Clark and Baro, 2006). While receptor-G protein interactions are not mediated solely by this structural feature, the extreme conservation suggests that the mechanisms for receptor-G protein interactions will be similar in mammals and arthropods. This predicts that arthropodal GPCRs will activate the same G protein(s) as homologous mammalian receptors when expressed in mammalian cell lines. Mammalian type-2 DARs stimulate both PTX sensitive ($G\alpha_o$, $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$) and insensitive

Table 1
Alternate splicing of $D_{2\alpha\text{Pan}}$

Location	Isoform	Exon configuration	Amino acid sequence ^{1,2}
Intracellular loop 3	$D_{2\alpha\text{Pan}.1}$	+i3 alternately spliced exon	...qdeeEEEGEDVMGLGGeenc...
	$D_{2\alpha\text{Pan}.2}$	+i3 alternately spliced exon	...qdeeEEEGEDVMGLGGeenc...
	$D_{2\alpha\text{Pan}.3}$	-i3 alternately spliced exon	...qdeeeenc...
	$D_{2\alpha\text{Pan}.4}$	-i3 alternately spliced exon	...qdeeeenc...
C-terminus	$D_{2\alpha\text{Pan}.1}$	$D_{2\alpha}$ C-terminal exon 1	...ilisiqS*
	$D_{2\alpha\text{Pan}.4}$	$D_{2\alpha}$ C-terminal exon 1	...ilisiqS*
	$D_{2\alpha\text{Pan}.2}$	$D_{2\alpha}$ C-terminal exon 2	...ilisiqMTISSNSFSLETVVLENHASC*
	$D_{2\alpha\text{Pan}.3}$	$D_{2\alpha}$ C-terminal exon 2	...ilisiqMTISSNSFSLETVVLENHASC*

¹The * represents a stop codon.

²Capital letters represent alternately spliced exons, lower case letters represent amino acids present in all isoforms examined.

(G_{α_z}) members of the $G_{\alpha_{i/o}}$ family (Obadiah et al., 1999; Banihashemi and Albert, 2002; Ghahremani et al., 1999). We stably expressed the $D_{2\alpha.1Pan}$ construct in a HEK cell line (HEKD $_{2\alpha.1Pan}$) and performed our previously described G protein activation assay (Clark and Baro, 2006) to determine receptor-G protein coupling. Fig. 2 illustrates that $D_{2\alpha.1Pan}$ couples with both PTX sensitive and PTX insensitive members of the $G_{\alpha_{i/o}}$ family, but not with G_{α_s} , G_{α_q} , or $G_{\alpha_{12}}$. The receptor appeared to couple most strongly with $G_{\alpha_{i3/o}}$, producing a significant 2.4-fold increase in activity in response to a 15-min application of 10 μ M DA ($p < 0.007$). There was also significant coupling with G_{α_z} (1.8-fold increase in activity, $p < 0.02$) and $G_{\alpha_{i1/2}}$ (1.4-fold increase in activity, $p < 0.02$).

3.3. DA activates $D_{2\alpha.1Pan}$ to produce an increase in cAMP

$D_{2\alpha Pan}$ orthologs have been shown to respond to multiple monoamines. In addition to DA, tyramine stimulates the DD2R and *AmDop3* receptors, and DD2R responds to serotonin (5-HT) (Hearn et al., 2002; Beggs et al., 2005). $G_{\alpha_{i/o}}$ proteins are known to decrease AC activity and reduce [cAMP]_i. We therefore further characterized the $D_{2\alpha.1Pan}$ receptor by measuring [cAMP]_i in cells after a 30 min exposure to one of five monoamines that are endogenous to lobster nervous tissue. Fig. 3 illustrates that at a concentration of 1mM, 5-HT, octopamine, tyramine and histamine had no significant effect on [cAMP]_i in HEKD $_{2\alpha.1Pan}$ relative to parental cells. On the other hand, DA produced a significant 2.3-fold increase in [cAMP]_i in HEKD $_{2\alpha.1Pan}$ relative to parental cells ($p < 3 \times 10^{-4}$). Collectively, these data suggest that DA is the only endogenous monoamine that activates the $D_{2\alpha.1Pan}$ receptor when expressed in HEK cells.

3.4. The DA-induced increase in [cAMP]_i is mediated by Gi/o proteins

Interestingly, DA produced an increase in cAMP levels (Fig. 3), despite the fact that the $D_{2\alpha.1Pan}$ receptor couples with the Gi/o family (Fig. 2). This is contrary to previous studies on the fly and bee orthologs of $D_{2\alpha Pan}$, which show that when these receptors are expressed in HEK cell lines, exposure to DA produces a decrease in cAMP (Beggs et al., 2005; Hearn et al., 2002). It is not clear whether the difference lies in the cell lines or the arthropod D_2 receptors. In order to elucidate the mechanism responsible for this difference, we further characterized the lobster D_2 signaling cascade(s) in HEK cells.

DARs can signal through mechanisms independent of the traditional, G protein mediated pathways (Beaulieu et al., 2005; Zou et al., 2005; Lee and Liu, 2004; Pei et al., 2004; Liu et al., 2000; Lefkowitz and Shenoy, 2005). To determine if the DA-induced increase in cAMP is due to Gi/o proteins (Fig. 2) and/or G protein independent cascades, we examined the effect of DA in the presence of PTX, which specifically blocks the activation and dissociation of all members of the Gi/o family, except Gz. We hypothesized that if PTX can partially block the DA-induced increase in cAMP, it would suggest that Gi/o proteins help to mediate the response.

Fig. 4 (solid line) shows that HEKD $_{2\alpha.1Pan}$ cells produced a dose-dependent increase in cAMP levels in response to DA, with an EC_{50} of 9.2×10^{-7} M, while the parental HEK cell line did not respond to DA. The dashed line illustrates that application of PTX significantly attenuated the DA response, and reduced the maximal fold change in cAMP from 4.4 to 3 ($p < 0.0008$). On the other hand, PTX had no effect on cAMP levels in the parental HEK cell line. These data, along with Fig. 2, suggest that the DA-evoked increase in cAMP depends, in part, on PTX sensitive trimeric Gi/o proteins. The DA response was not completely eliminated despite the fact that a saturating concentration of PTX was applied (500 ng/mL; see Fig. 4 inset for PTX dose-response curve). This is at least partially due to coupling between the $D_{2\alpha.1Pan}$ receptor and the PTX insensitive Gz protein (Fig. 2); however, we cannot rule out the possibility that the $D_{2\alpha Pan}$ receptor activates additional G protein independent cascades to increase [cAMP]_i.

3.5. The $G\beta\gamma$ subunits of Gi/o proteins contribute to DA-induced alterations in [cAMP]_i

AC can be regulated by both G_{α} and $G\beta\gamma$ subunits (Federman et al., 1992). While $G_{\alpha_{i/o}}$ subunits decrease or have no effect on AC activity, $G\beta\gamma$ can increase or decrease AC activity depending on the AC and $G\beta\gamma$ isozymes involved (Cabrera-Vera et al., 2003). We tested the hypothesis that $G\beta\gamma$ subunits mediate the DA-induced increase in [cAMP]_i in the HEKD $_{2\alpha.1Pan}$ cell line by blocking the $G\beta\gamma$ pathway with known $G\beta\gamma$ scavengers. Dexas1 has been shown to

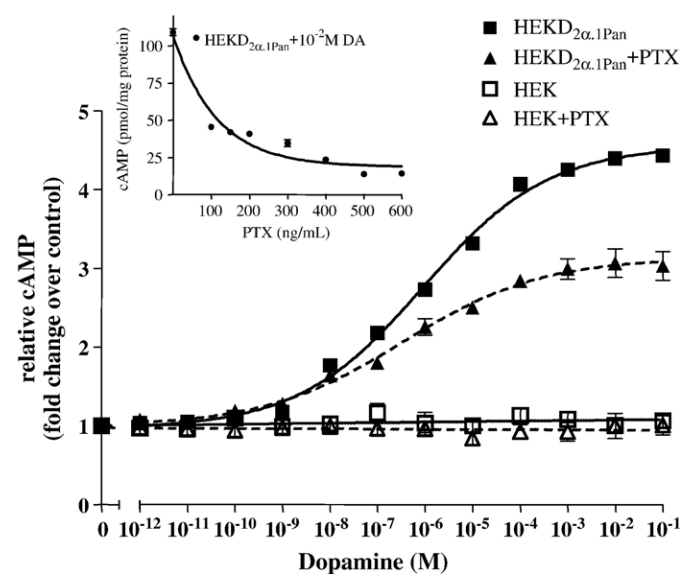


Fig. 4. The $D_{2\alpha.1Pan}$ receptor couples positively with cAMP through PTX sensitive Gi/o proteins, as well as PTX insensitive cascades. [cAMP] was simultaneously measured in pmol cAMP/mg protein for both HEKD $_{2\alpha.1Pan}$ (solid symbols) and HEK (open symbols) cells in the presence of increasing DA. All data points are normalized to [cAMP] measured in the absence of DA (control) in the same experiment, and the fold change over control is plotted on the y axis. Experiments were conducted in the presence (dashed line) vs. the absence (solid line) of the Gi/o inhibitor, PTX (500 ng/mL). The inset represents a PTX dose-response curve for the HEK $D_{2\alpha.1Pan}$ cell line. All experiments were repeated 3 times. Data represent the mean \pm S.E.M.

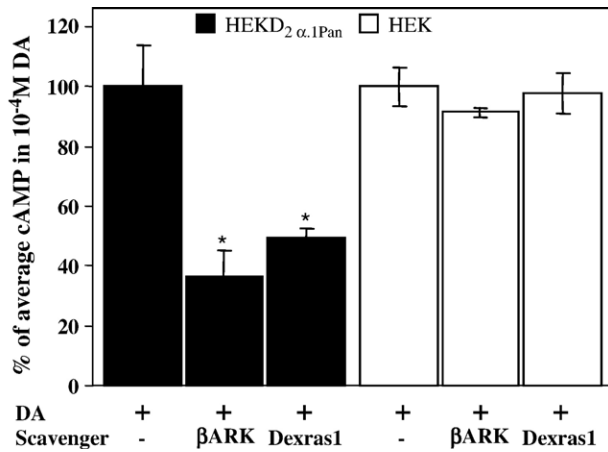


Fig. 5. The increase in cAMP in HEKD_{2α.1Pan} is mediated by Gi/o βγ subunits. The Gβγ scavengers dexas1 or βARK_{495–689} were transiently expressed in HEKD_{2α.1Pan} (black bars) and HEK (white bars) cells, as indicated below each bar. Cells were exposed to 10⁻⁴ M DA, and [cAMP] (pmol cAMP/mg protein) was measured. Data are normalized to the average [cAMP] in the presence of DA for each cell line. Data represent the mean ± S.E.M., *n* = 3. Student *t*-tests were performed, and asterisks indicate significant differences from cAMP levels measured in the absence of dexas1 or βARK_{495–689} within a cell line (*p* < 0.04).

specifically block agonist-stimulated GPCR activation of Gβγ signaling (Nguyen and Watts, 2005). Similarly, the carboxyl-terminal domain of βARK1 and βARK2 (βARK_{495–689}) suppresses Gβγ-mediated responses by scavenging free βγ subunits (Koch et al., 1994). We transiently expressed

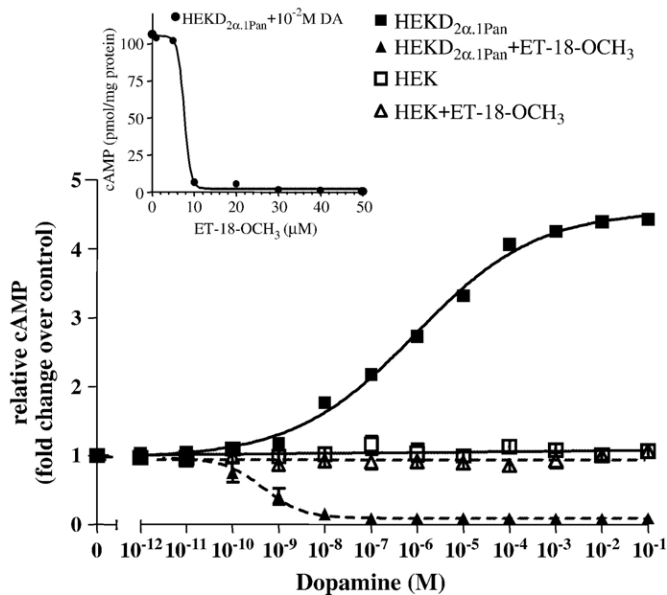


Fig. 6. Blocking PLCβ reveals a negative coupling between the D_{2α.1Pan} receptor and cAMP. [cAMP] was simultaneously measured in pmol cAMP/mg protein for both HEKD_{2α.1Pan} (solid symbols) and HEK (open symbols) cells in the presence of increasing DA. All data points are normalized to [cAMP] measured in the absence of DA (control) in the same experiment, and the fold change over control is plotted on the *y* axis. Experiments were conducted in the presence (dashed line) vs. the absence (solid line) of the PLCβ inhibitor, ET-18-OCH₃ (50 μM). The inset represents an ET-18-OCH₃ dose-response curve for the HEK D_{2α.1Pan} cell line. All experiments were repeated 3 times. Data represent the mean ± S.E.M.

βARK_{495–689} or dexas1 in HEK and HEKD_{2α.1Pan} cell lines and measured [cAMP]_i in the presence of 10⁻⁴ M DA. Fig. 5 shows that expression of either βARK_{495–689} or dexas1 significantly inhibited the DA-induced increase in cAMP in HEKD_{2α.1Pan} cells by 64 ± 9% (*p* < 0.04) and 51 ± 3% (*p* < 0.008) respectively, but had no significant effect on the parental cell line (*p* > 0.7). These data suggest that Gβγ subunits contribute to the DA-induced changes in [cAMP] in HEKD_{2α.1Pan} cells.

3.6. Blocking the Gβγ cascade reveals a DA-induced, Gα_{i/o} mediated decrease in cAMP

Gβγ can have many immediate effectors, including PLCβ, ACs, ion channels, kinases and components of the synaptic vesicle release machinery (Blackmer et al., 2005; Cabrera-Vera et al., 2003; Stehno-Bittel et al., 1995; Sullivan, 2005; Gerachshenko et al., 2005). It has been previously demonstrated that D₂ receptors can regulate ACII activity via Gβγ-mediated activation of PLCβ (Tsu and Wong, 1996). To determine whether Gβγ subunits act via PLCβ in HEKD_{2α.1Pan} cells, we applied the PLCβ inhibitor, ET-18-OCH₃, and measured cAMP levels in the presence of increasing concentrations of DA. Fig. 6 (dashed line) demonstrates that inhibiting PLCβ also inhibited the DA-induced increase in cAMP, and revealed a dose-dependent decrease in cAMP.

The dose-dependent decrease in cAMP was largely PTX sensitive. Fig. 7 illustrates that in the presence of forskolin (an AC activator) and ET-18-OCH₃, DA evokes a clear dose-dependent decrease in cAMP in HEKD_{2α.1Pan} cells with an

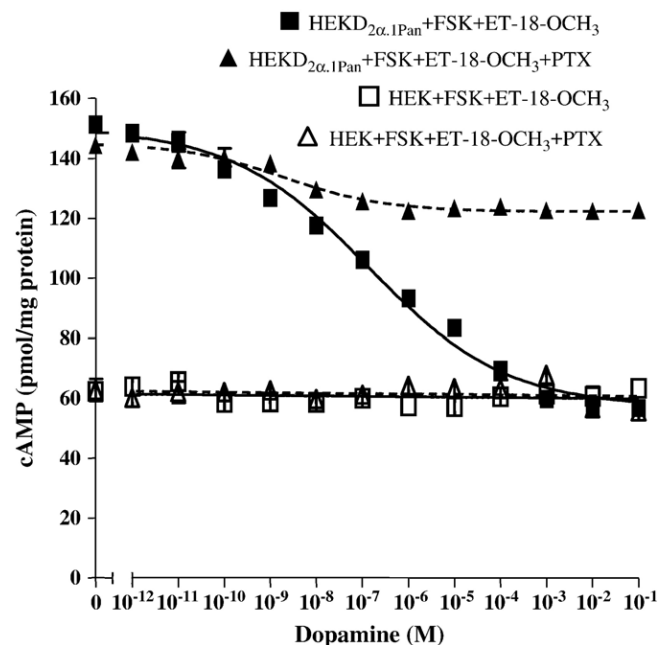


Fig. 7. The negative coupling to cAMP is mediated by PTX sensitive and PTX insensitive G proteins. All cells were exposed to forskolin (2.5 μM) and ET-18-OCH₃ (50 μM). [cAMP] was simultaneously measured in pmol cAMP/mg protein for both HEKD_{2α.1Pan} (solid symbols) and HEK (open symbols) cells in the presence of increasing DA. Experiments were conducted in the presence (dashed line) vs. the absence (solid line) of the Gi/o inhibitor, PTX (500 ng/mL). All experiments were repeated 3 times. Data represent the mean ± S.E.M.

EC_{50} of 1.4×10^{-7} M. The total inhibition by saturating levels of PTX was 77% of the maximal response. The response that remained in the presence of PTX was most likely mediated by Gz (Fig. 2). Collectively, these data suggest that DA initiates parallel signaling cascades in HEKD_{2 α .1Pan} cells with opposing effects on cAMP levels: the G $\alpha_{i/o}$ subunits cause a decrease in cAMP while G $\beta\gamma$ subunits activate PLC β to cause an increase in cAMP.

Fig. 7 also suggests that the D_{2 α .1Pan} receptor, like its mammalian and *Drosophila* homologs, may constitutively activate Gi/o proteins. The forskolin activated cAMP levels are significantly higher in HEKD_{2 α .1Pan} relative to the parental HEK cell line (Fig. 7; 150 pmol/mg vs. 60 pmol/mg; $p < 10^{-4}$). This compensatory mechanism is known as heterologous sensitivity or supersensitivity (Watts, 2002; Vortherms et al., 2004; Watts and Neve, 2005). Several studies have demonstrated that chronic Gi/o activity ultimately leads to a paradoxical increase in AC activity (supersensitivity) through a number of different molecular mechanisms.

3.7. The intracellular milieu determines whether arthropod D₂ receptors positively or negatively couple with cAMP

As previously stated, when expressed in HEK293 cells, the lobster versus fruit fly and honeybee orthologs of the D₂ receptor produce opposite changes in cAMP levels in response to DA: the lobster D₂ receptor positively couples with cAMP (Fig. 3) while the fly and bee orthologs of the D₂ receptor negatively couple with cAMP (Beggs et al., 2005; Hearn et al., 2002). We predicted that if the cellular background determines whether D₂ receptors positively or negatively couple with cAMP, then expressing the honeybee ortholog of the D₂ receptor, *AmDop3*, in our parental HEK293 cell line should produce an increase, rather than the previously described decrease in cAMP. To test the hypothesis we obtained the *AmDop3* clone from the Mercer lab, transformed our HEK293 cells to generate a stable cell line, HEK*AmDop3*, and measured changes in cAMP in response to DA. Fig. 8 shows that the HEK*AmDop3* cells responded to 10^{-4} M DA with a significant increase in cAMP ($p < 0.0001$). The DA-induced increase in HEK*AmDop3* cells is roughly 7-fold greater than that observed in the parental HEK line ($p < 0.009$, HEK*AmDop3* vs. HEK). The increase was attenuated by transiently expressing the G $\beta\gamma$ scavenger β ARK_{495–689}. Together these data suggest that the cellular milieu greatly influences D₂ mediated changes in [cAMP] and that there are no obvious functional differences between the signaling properties of the honeybee and lobster D₂ orthologs.

3.8. Alternate splicing changes of the potency and efficacy of D_{2 α Pan} isoforms

Fig. 1 and Table 1 indicate that the D_{2 α Pan} receptor can be alternately spliced to create multiple isoforms with differences in their carboxy termini and/or intracellular loop 3. The carboxy terminus and intracellular loop 3 are involved in G protein coupling (Wong, 2003). Changes in amino acid sequence in these

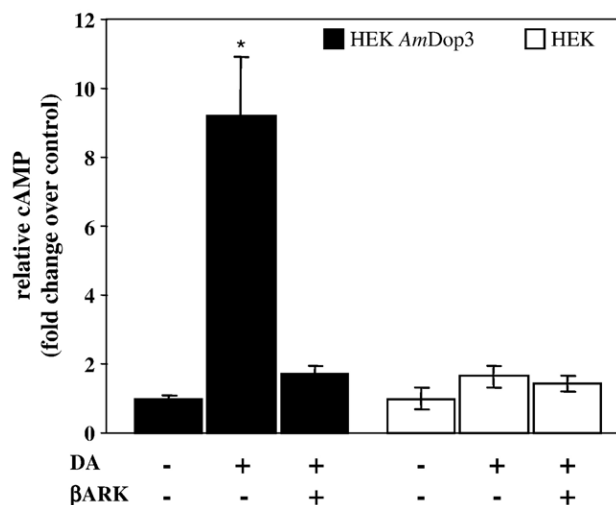


Fig. 8. *AmDop3* positively couples to cAMP through the G $\beta\gamma$ cascade. Levels of cAMP (pmol cAMP/mg protein) were simultaneously measured in HEK cells expressing the *AmDop3* receptor (HEK*AmDop3*, black bars) and in the parental HEK cell line (white bars) in the presence or absence of 10^{-4} M DA and in the presence or absence of the G $\beta\gamma$ scavenger β ARK_{495–689}, as indicated below each bar. All data were normalized to average [cAMP] in the absence of DA (control) for the same experiment, and fold change over control is plotted on the y axis. All experiments were repeated 3 times. Data represent the mean \pm S.E.M. Data were analyzed using Student *t*-tests. * Indicates significant differences from cAMP levels measured in the control for the same cell line ($p < 0.0001$).

regions can alter the strength or specificity of G protein signaling (Franke et al., 1990; Cotecchia et al., 1990). The C-terminus of GPCRs also determines the rate of receptor recycling and receptor coupling to β -arrestin mediated cascades (Oakley et al., 1999). To determine whether alternate splicing produces functional differences in G protein signaling, we established stable HEK cell lines expressing D_{2 α .2Pan} or D_{2 α .3Pan} and obtained DA dose-response curves for the resulting cell lines: HEKD_{2 α .2Pan} and HEKD_{2 α .3Pan}. Fig. 9A shows that in all cases the receptor produces a dose-dependent increase in [cAMP]_i that is significantly higher than in the parental HEK cell line (2.5-fold and 2-fold greater, respectively, at 10^{-4} M DA; $p < 0.05$). Altering the carboxy terminal domain (D_{2 α .1Pan} vs. D_{2 α .2Pan}) reduced the EC_{50} by more than an order of magnitude (from 9.2×10^{-7} M to 2.4×10^{-6} M, respectively). In addition, removing the alternately spliced intracellular loop 3 exon (D_{2 α .2Pan} vs. D_{2 α .3Pan}) once again changed the EC_{50} by more than an order of magnitude (from 2.4×10^{-6} to 4×10^{-7} , respectively) and significantly altered receptor efficacy. Thus, these data suggest that alternate splicing may change the potency of the D_{2 α Pan} receptor. We also tested the effect of 5HT, octopamine, tyramine, and histamine on [cAMP]_i in HEKD_{2 α .2Pan} and HEKD_{2 α .3Pan} cell lines. Fig. 9B shows that, like the D_{2 α .1Pan} receptor (Fig. 3), D_{2 α .2Pan} and D_{2 α .3Pan} respond only to DA when expressed in HEK cells. Thus, alternate splicing does not affect the monoamine specificity of these receptors.

4. Discussion

CPGs are highly modulated neural circuits that rely on GPCRs to produce a rhythmic output (Ramirez et al., 2004;

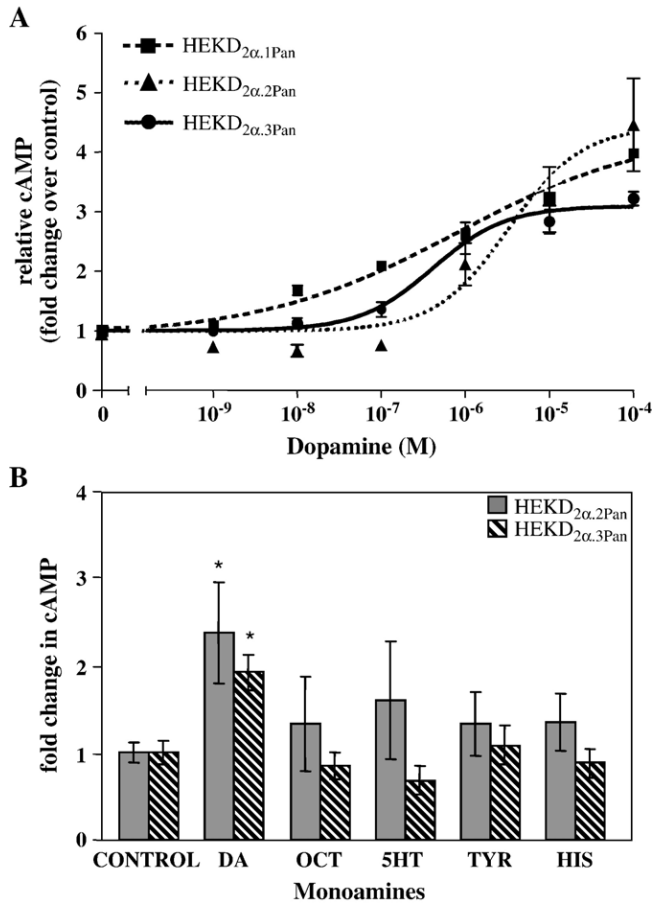


Fig. 9. Alternate splicing changes the potency and efficacy of $D_{2\alpha Pan}$ isoforms. A. Changes in cAMP levels (pmol cAMP/mg protein) in response to increasing [DA] were measured in HEKD_{2α.1Pan} (squares, dashed line) HEKD_{2α.2Pan} (triangles, dotted line) and HEKD_{2α.3Pan} (circles, solid line) cell lines. All data are normalized to [cAMP] in the absence of DA (control) and fold changes over control are plotted on the y axis. Data represent the mean ± S.E.M, $n=3$. B. Levels of cAMP (pmol cAMP/mg protein) were simultaneously measured in HEK, HEKD_{2α.2Pan} (grey bars) and HEKD_{2α.3Pan} (hatched bars) cells under control conditions (no monoamines present) or in the presence of 1mM of the indicated monoamine. Data were first normalized to control, and then the responses of the transfected cell lines were normalized to that of the parental HEK cell line. Average fold changes in the transfected relative to parental line are plotted, error bars indicate the S.E.M, $n>3$. * Indicates significant differences from cAMP levels measured in the control ($p<0.05$).

Marder and Bucher, 2001). The effects of DA on a model CPG, the pyloric network, have been extremely well characterized (Harris-Warrick et al., 1998; Gruhn et al., 2005; Johnson et al., 2003; Kloppenburg et al., 2000, 1999; Peck et al., 2001); however, the molecular mechanisms by which DA exerts its effects are completely unknown. To begin to investigate the molecular underpinnings of the dopaminergic response in pyloric neurons, we cloned and characterized the only known arthropod type-2 DAR from *P. interruptus*: $D_{2\alpha Pan}$. Heterologous expression in HEK cells indicates that this receptor is specifically activated by DA, as opposed to other monoamines known to be endogenous to the lobster nervous system. Alternate splicing in intracellular loop 3 and at the carboxy terminus alters the potency and efficacy of the receptor. Surprisingly, we found that when expressed in HEK cells the

$D_{2\alpha Pan}$ receptor positively couples with cAMP. The increase in cAMP is mediated, in part, by multiple Gi/o proteins. $D_{2\alpha Pan}$ stimulation of Gi/o activity results in the activation of two discrete pathways: 1) a $G\alpha$ mediated inhibition of AC, leading to a decrease in cAMP and 2) a $G\beta\gamma$ -mediated activation of PLC β , leading to an increase in cAMP. We also found that contradictory to previous reports (Beggs et al., 2005), the honeybee D_2 receptor can positively couple with cAMP via the $G\beta\gamma$ subunits of Gi/o proteins, suggesting that the intracellular environment can alter receptor coupling to cAMP. We conclude that arthropod and mammalian D_2 receptor signaling is very similar, and that D_2 mediated signaling is determined by both the functional properties of the receptor and the intracellular milieu.

4.1. The $D_{2\alpha Pan}$ receptor simultaneously activates multiple cascades

It is not clear whether the $D_{2\alpha Pan}$ receptor response is mediated entirely by G proteins in HEK cells. Fig. 7 suggests that a PTX insensitive protein, probably $G\alpha_z$, mediates roughly 23% of the DA-induced decrease in cAMP while the PTX sensitive $G\alpha_{i/o}$ subunits are responsible for 77% of the response. However, saturating levels of PTX only reduced the DA-induced increase in cAMP from 4.4- to 3-fold, rather than the predicted 1.8-fold (Fig. 4). Furthermore, the EC_{50} for the increase in cAMP (9.2×10^{-7} ; Fig. 4) is 6.6-fold higher than the EC_{50} for the decrease in cAMP (1.4×10^{-7} ; Fig. 7). There are at least two possible explanations for these findings, and they are not mutually exclusive. First, $D_{2\alpha Pan}$ receptors may simultaneously activate multiple cascades, including G protein independent cascades (Beaulieu et al., 2005; Lefkowitz and Shenoy, 2005). Second, $G\alpha_z$ may donate the majority of $G\beta\gamma$ subunits that interact with PLC β to increase cAMP. Specific $G\alpha$ donors for $G\beta\gamma$ subunits have previously been observed in certain cell types. For example, GIRK channels are activated by $G\beta\gamma$ subunits that are exclusively donated by $G\alpha_{12}$ and $G\alpha_{13}$ in native tissues, though any $G\alpha$ subunit can donate the $G\beta\gamma$ subunits in studies utilizing heterologous expression systems (Dascal, 2001). Specificity in native tissues appears to be conferred by binding of the α -subunit to the GIRK effector (Ivanina et al., 2004) and the fact that upon activation, Gi and Gz proteins undergo a conformational change, but do not dissociate into physically independent $G\alpha$ and $G\beta\gamma$ subunits (Frank et al., 2005). Although $G\alpha$ donor specificity has never to our knowledge been observed for $G\beta\gamma$ regulation of PLC β , we cannot dismiss this concept a priori.

Unexpectedly, the $D_{2\alpha Pan}$ receptor-initiated cascades regulate cAMP in opposing directions in the same cells. These cascades may be highly localized to create microdomains of cAMP gradients (Zaccolo and Pozzan, 2002; Rich et al., 2001). On the other hand, the cascades may function with different kinetics and interact to generate feedback loops. In addition, there are examples of G protein mediated cascades dominating the early portion of a response to constant agonist application, while β -arrestin cascades predominate in later portion (Ahn et al., 2004). Thus, distinct $D_{2\alpha Pan}$ mediated

cascades may operate in different timeframes to generate multiphasic responses.

4.2. Receptor signaling varies with the intracellular milieu

Interestingly, a D_2 receptor can produce opposite responses even when expressed in the “same” cell type. When *AmDop3*, the honeybee ortholog of the arthropod D_2 receptor, is expressed in HEK293 cells in the Mercer lab, it produces a decrease in cAMP; however, when it is expressed in HEK293 cells in the Baro lab, it produces an increase in cAMP. Such a finding is not unique to the arthropod D_2 receptor. For example, isoproterenol induced β_2 -adrenergic receptor signaling in HEK293 cells varies across labs (Daaka et al., 1997; Friedman et al., 2002; Lefkowitz et al., 2002). Tissue culture cell lines can often rearrange their genetic material and/or alter their genetic programs, most likely because culture conditions provide little selective pressure for maintaining a constant genome/transcriptome/proteome. Thus, receptor signaling in a given cell type may vary with the lab because cell lines diverge within and across labs over time. Indeed, in our hands the parental HEK cell line could alter its response to DA, despite the fact that it was cultured under constant conditions. Differences in the expression, localization and/or interactions of downstream effectors of the D_2 receptor could account for the differences in the *AmDop3* response in each HEK cell line. All of these findings reinforce the idea that GPCR signaling is context dependent. Based on these studies, we cannot predict how the $D_{2\alpha\text{Pan}}$ receptor will affect cAMP levels in pyloric neurons; though the data suggest that the $D_{2\alpha\text{Pan}}$ receptor will most likely couple with Gi and Go proteins to alter cAMP levels in pyloric neurons.

4.3. Monoaminergic GPCR signaling is conserved across species

Relatively little is known about invertebrate monoaminergic GPCRs compared to their vertebrate homologs. Data mining studies suggest that there are roughly 19 monoamine receptors in arthropods (Clark et al., 2004; Roeder, 2003). By the year 2004, 10 of these receptors had been cloned and characterized (Tierney, 2001; Blenau and Baumann, 2001; Clark et al., 2004). Several recent efforts have reduced the number of uncharacterized monoaminergic receptors to roughly 3 out of 19 (Balfanz et al., 2005; Srivastava et al., 2005; Maqueira et al., 2005; Cazzamali et al., 2005; Evans and Maqueira, 2005).

Both receptors and G proteins show strong amino acid sequence conservation in functional domains across species. Here we have demonstrated that the arthropod D_2 receptor can regulate second messenger levels by coupling to both AC, via $G\alpha$ subunits, and PLC β , via $G\beta\gamma$ subunits of Gi/o proteins. Similar findings were previously published for mammalian D_2 receptors expressed in HEK293 cells (Tsu and Wong, 1996) and in native neurons (Hernandez-Lopez et al., 2000). In addition, we have previously shown that comparable to mammalian type-1 DARs, the $D_{1\alpha\text{Pan}}$ receptor couples with Gs and the $D_{1\beta\text{Pan}}$

receptor couples with both Gs and Gz when expressed in HEK cells (Clark and Baro, 2006). Likewise, we have shown that the $5\text{-HT}_{2\beta\text{Pan}}$ receptor couples with Gq (Clark et al., 2004) and the $5\text{-HT}_{1\alpha\text{Pan}}$ receptor couples with Gi/o (Spitzer and Baro, submitted), as is the case for their respective mammalian homologs. Collectively, these data strongly suggest that signaling mechanisms for homologous receptors are well conserved across species.

5. Conclusion

Receptor expression studies in heterologous systems are important as they help to define key structure/function relationships for homologous receptors across species. Such studies are also useful and necessary in that they reveal organizing principles for signal transduction and more specifically, the repertoire of cascades available to a given receptor. However, heterologous expression studies are limited by the fact that receptor signaling is context dependent. In order to understand the function of a receptor in a specific cell type, the receptor must ultimately be studied in that cell type. We have found that when the $D_{2\alpha\text{Pan}}$ receptor is heterologously expressed, it couples with Gi/o proteins and can modulate cAMP levels through both $G\alpha$ and $G\beta\gamma$ subunits, like all of its homologs. The data also suggest that $D_{2\alpha\text{Pan}}$ receptor signaling may involve additional Gi/o-independent mechanisms. These results set the stage for future studies aimed at understanding the role of D_2 receptors in native neurons involved in rhythmic motor pattern generation.

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