Published in final edited form as:
Comp Biochem Physiol B Biochem Mol Biol. 2007 January ; 146(1): 9–19.

Arthropod D_2 receptors positively couple with cAMP through the Gi/o protein family

Merry C. Clark and **Deborah J. Baro**
Program for Cell and Molecular Biology and Physiology, Department of Biology, Georgia State University, P.O. Box 4010, Atlanta, Georgia 30303, USA

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 D_2 receptor, $D_{2\alpha Pan}$. We found that when expressed in HEK cells, the $D_{2\alpha Pan}$ 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 Gα mediated inhibition of adenylyl cyclase (AC), leading to a decrease in cAMP and 2) a Gβγ mediated activation of Phospholipase Cβ (PLCβ), 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 D_2 receptor coupling with cAMP varies with the cellular milieu.

Keywords

Central pattern generator; Crustacean; G protein coupled receptor; Heterologous expression; Signal transduction; Stomatogastric

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;Harris-Warrick et al., 1995a;Harris-Warrick et al., 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).

Correspondence to: Deborah J. Baro, Biology Department, Georgia State University, P.O. Box 4010, Atlanta, GA 30303, USA, phone: (404) 651-3107; fax: (404) 651-2509, email: dbaro@gsu.edu.

In the traditional view, DARs are classified as type-1 or type 2: type-1 DARs couple to Gs proteins, leading to a Gα-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;Sidhu et al., 1998;Zheng et al., 2003;O'Sullivan et al., 2004;Zhen et al., 2004;Kimura et al., 1995b). 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 Gα and Gβγ 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 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, $D_{2\alpha Pan}$.

MATERIALS AND METHODS

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 (Figure 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 *Am*Dop3 constructs were stably expressed in HEK293 cells using methods previously described (Clark et al., 2004). *Am*Dop3 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 Gβγ scavengers, dexras1 (UMR cDNA resource center, University of Missouri-Rolla) or $\beta ARK_{495–689}$ (kindly provided by Dr. Robert Lefkowitz, Howard Hughes Medical Institute), were transiently expressed. In these cases, cells were maintained in DMEM supplemented with 10% dialyzed fetal bovine serum plus 600μg/mL neomycin ($HEKD_{2\alpha Pan}$ or HEK*Am*Dop3) or 50 units/mL penicillin and 50μg/mL streptomycin (parental HEK cells) at 37°C, 5% CO_2, and were grown to 90–95% confluency in 26x33mm 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 10μL lipofectamine in 100uL

opti-MEM according to the manufacturer's instructions. After 6 hours at 37°C, 5% CO_2, cells received 1mL of DMEM containing 20% dialyzed serum. Cells received normal media (with antibiotic) 24 hours following transfection, and were assayed 24–48 hours 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 100mM. The assays shown in Figures 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 $HEKD_{2\alpha.1Pan}$ cells were thawed and the assays shown in Figures 3 and 5 were performed. In addition, the parental line was also transfected to generate stable HEK*Am*Dop3, $HEKD_{2\alpha.2Pan,}$ and $HEKD_{2\alpha.3Pan}$ lines, and the assays shown in Figures 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 D_1 receptor that in some assays produced a significant increase in cAMP in response to 10^{-4}M DA or the D_1 selective agonist, 6-chloro-PB (n= 3, $p< 0.05$). The pharmacology of the human D_1 receptor was distinct from the $D_{2\alpha Pan}$ receptor. The $D_{2\alpha Pan}$ receptor produced an increase in cAMP in response to 10-5M quinpirole (n=3, $p < 0.05$), a selective D_2 agonist, while the parental D_1 receptor did not (n=3, $p> 0.05$). Furthermore, the signaling properties of the two receptors were distinct: The arthropod D_2 receptor relies on the Gβγ subunit to produce an increase in cAMP while the human D_1 receptor does not (Figures 5 and 8).

Membrane preparations

Stably transfected cells were harvested with trypsin (ATCC, Manassas, VA). Pellets were homogenized in 20mM HEPES (pH 7.4) containing 2mM $MgCl_2$, 1 mM EDTA, 2mM 1,4-dithiothreitol (DTT), 1μg/mL leupeptin, 1μg/mL aprotinin, and 2mM PMSF. The homogenate was centrifuged at 2500 rpm for 5 minutes. The supernatant was recovered and centrifuged at 15,000 rpm for 30 minutes at 4°C. Pellets were resuspended in 20mM HEPES (pH 7.4) containing 0.5% 3-[(3 cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and 2mM EDTA. Protein concentrations in each sample were determined using a BCA Protein Assay Kit (Pierce).

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 (1.5μg/ul of protein) were incubated at 37°C for 15 minutes in 10mM HEPES (pH 7.4) containing 10mM $MgCl_2$, 100μM EDTA and 10nM GTPγ^{35}S (Amersham) with or without DA. Reactions were terminated with ten volumes of termination buffer [10mM $MgCl_2$, 100μM GDP, 200mM NaCl in 100mM Tris (pH 8.0)]. Fifty μl of each terminated sample were then aliquotted in triplicate to wells precoated with one antibody against a human Gα subunit [$G_{12}α$, $G_{i1/2}α$, $G_qα$, $G_{i3/o}α$, $G_sα$, or $G_zα$ (EMD/Calbiochem)] and to uncoated wells (blanks). Plates were incubated on ice for 2 hours. 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 nonspecific binding was subtracted from the average cpm obtained from the coated wells. Data are expressed as cpm/μg of protein.

cAMP assays

cAMP levels were measured as previously described (Clark et al., 2004). Briefly, 1×10^5 cells were plated in 35mm dishes and grown to confluence. Cells were washed with 1 mL of media and preincubated at 37°C for 10 minutes in the presence of the phosphodiesterase inhibitor 3-

isobutyl-1-methylxanthine (2.5mM) (Sigma). Cells were incubated an additional 30 minutes at 37°C with or without forskolin (2.5μM), and varying concentrations of monoamine (DA, 5-HT, tyramine, histamine, or octopamine). In some experiments, cells were pretreated for 24 hours with pertussis toxin (PTX, Calbiochem) or 15 minutes with 1-O-Octadecyl-2-O-methyl-rac-glycero-3-phosphorylcholine (Et-18-OCH_3, Calbiochem). The media was removed and 0.5mL of 0.1M HCl with 0.8% Triton X-100 (Sigma) was added to the plates. After a 30 minute incubation at room temperature, the lysate was removed from the plates and centrifuged for 2 minutes. 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).

Statistical analyses and curve fitting

Student t-tests were performed with Excel software. Curve fitting, Kruskall-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 $p < 0.05$.

RESULTS

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, *Panulirus interruptus*. We found that this receptor, $D_{2\alpha Pan}$, is alternately spliced (see arrowheads in Figure 1) to produce four distinct proteins: $D_{2\alpha.1Pan}$, $D_{2\alpha.2Pan}$, $D_{2\alpha.3Pan}$, and $D_{2\alpha.4Pan}$ (Table 1). We did not conduct an exhaustive search for $D_{2\alpha Pan}$ isoforms, and it is likely that additional splice forms exist (Hearn et al., 2002).

The $D_{2\alpha Pan}$ 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 sapien* Reference Proteins database showed that the $D_{2\alpha Pan}$ receptor was most homologous to the long form of the human D_2 receptor (NP_000786) with an E value of 3e-58. Figure 1 illustrates that all D_2 receptors are well conserved across species. When compared to its fly, honeybee and human homologs, the $D_{2\alpha Pan}$ 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 (Figure 1).

$D_{2\alpha.1Pan}$ 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 Gα 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 ($G\alpha_z$) 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. Figure 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 $G\alpha_s$, $G\alpha_q$, 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-minute application of 10μM DA ($p < 0.007$). There was also significant coupling with $G\alpha_z$ (1.8-fold increase in activity, $p < 0.02$) and $G\alpha_{i1/2}$ (1.4-fold increase in activity, $p < 0.02$).

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 *Am*Dop3 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 minute exposure to one of five monoamines that are endogenous to lobster nervous tissue. Figure 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.

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

Interestingly, DA produced an increase in cAMP levels (Figure 3), despite the fact that the $D_{2\alpha.1Pan}$ receptor couples with the Gi/o family (Figure 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 (Figure 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.

Figure 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 Figure 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 Figure 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 (Figure 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.

The Gβγ subunits of Gi/o proteins contribute to DA-induced alterations in [cAMP]i

AC can be regulated by both Gα and Gβγ subunits (Federman et al., 1992). While $G\alpha_{i/o}$ subunits decrease or have no effect on AC activity, Gβγ can increase or decrease AC activity depending on the AC and Gβγ isozymes involved (Cabrera-Vera et al., 2003). We tested the hypothesis that Gβγ subunits mediate the DA-induced increase in [cAMP]i in the $HEKD_{2\alpha.1Pan}$ cell line by blocking the Gβγ pathway with known Gβγ scavengers. Dexras1 has been shown to specifically block agonist-stimulated GPCR activation of Gβγ signaling (Nguyen and Watts, 2005). Similarly, the carboxyl-terminal domain of βARK1 and βARK2 ($\beta ARK_{495–689}$) suppresses Gβγ-mediated responses by scavenging free βγ subunits (Koch et al., 1994). We transiently expressed $\beta ARK_{495–689}$ or dexras1 in HEK and $HEKD_{2\alpha.1Pan}$ cell lines and measured [cAMP]i in the presence of 10^{-4}M DA. Figure 5 shows that expression of either $\beta ARK_{495–689}$ or dexras1 significantly inhibited the DA-induced increase in cAMP in $HEKD_{2\alpha.1Pan}$ 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 $HEKD_{2\alpha Pan}$ cells.

Blocking the Gβγ cascade reveals a DA-induced, $G\alpha_{i/o}$ 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 D_2 receptors can regulate ACII activity via Gβγ mediated activation of PLCβ (Tsu and Wong, 1996). To determine whether Gβγ subunits act via PLCβ in $HEKD_{2\alpha Pan}$ cells, we applied the PLCβ inhibitor, ET-18-OCH_3, and measured cAMP levels in the presence of increasing concentrations of DA. Figure 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. Figure 7 illustrates that in the presence of forskolin (an AC activator) and ET-18-OCH_3, DA evokes a clear dose dependent decrease in cAMP in $HEKD_{2\alpha.1Pan}$ cells with an 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 $HEKD_{2\alpha.1Pan}$ cells with opposing effects on cAMP levels: the $G\alpha_{i/o}$ subunits cause a decrease in cAMP while Gβγ subunits activate PLCβ to cause an increase in cAMP.

Figure 7 also suggests that the $D_{2\alpha.1Pan}$ receptor, like its mammalian and *Drosophila* homologs, may constitutively activate Gi/o proteins. The forskolin activated cAMP levels are significantly higher in $HEKD_{2\alpha.1Pan}$ relative to the parental HEK cell line (Figure 7; 150pmol/mg vs. 60pmol/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.

The intracellular milieu determines whether arthropod D_2 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 D_2 receptor produce opposite changes in cAMP levels in response to DA: the lobster D_2 receptor positively couples with cAMP (Figure 3) while the fly and bee orthologs of the D_2 receptor negatively couple with cAMP (Beggs et al., 2005;Hearn et al., 2002). We predicted that if the cellular background determines whether D_2 receptors positively or negatively couple with cAMP, then expressing the honeybee ortholog of the D_2 receptor,

*Am*Dop3, 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 *Am*Dop3 clone from the Mercer lab, transformed our HEK293 cells to generate a stable cell line, HEK*Am*Dop3, and measured changes in cAMP in response to DA. Figure 8 shows that the HEK*Am*Dop3 cells responded to 10^{-4}M DA with a significant increase in cAMP ($p < 0.0001$). The DA-induced increase in HEK*Am*Dop3 cells is roughly 7-fold greater than that observed in the parental HEK line ($p < 0.009$, HEK*Am*Dop3 vs. HEK). The increase was attenuated by transiently expressing the Gβγ scavenger $\beta ARK_{495–689}$. Together these data suggest that the cellular milieu greatly influences D_2 mediated changes in [cAMP] and that there are no obvious functional differences between the signaling properties of the honeybee and lobster D_2 orthologs.

Alternate splicing changes the potency and efficacy of $D_{2\alpha Pan}$ isoforms

Figure 1 and Table 1 indicate that the $D_{2\alpha Pan}$ 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 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 $D_{2\alpha.2Pan}$ or $D_{2\alpha.3Pan}$ and obtained DA dose-response curves for the resulting cell lines: $HEKD_{2\alpha.2Pan}$ and $HEKD_{2\alpha.3Pan}$. Figure 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 ($D_{2\alpha.1Pan}$ vs. $D_{2\alpha.2Pan}$) 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 ($D_{2\alpha.2Pan}$ vs. $D_{2\alpha.3Pan}$) 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 $D_{2\alpha Pan}$ receptor. We also tested the effect of 5HT, octopamine, tyramine, and histamine on [cAMP]i in $HEKD_{2\alpha.2Pan}$ and $HEKD_{2\alpha.3Pan}$ cell lines. Figure 9B shows that, like the $D_{2\alpha.1Pan}$ receptor (Figure 3), $D_{2\alpha.2Pan}$ and $D_{2\alpha.3Pan}$ respond only to DA when expressed in HEK cells. Thus, alternate splicing does not affect the monoamine specificity of these receptors.

DISCUSSION

CPGs are highly modulated neural circuits that rely on GPCRs to produce a rhythmic output (Ramirez et al., 2004;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;Kloppenburg et al., 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 *Panulirus 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 Gα mediated inhibition of AC, leading to a decrease in cAMP and 2) a Gβγ mediated activation of PLCβ, 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 Gβγ 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.

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. Figure 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 (Figure 4). Furthermore, the EC_{50} for the increase in cAMP (9.2×10^{-7}; Figure 4) is 6.6-fold lower than the EC_{50} for the decrease in cAMP (1.4×10^{-7}; Figure 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 Gβγ subunits that interact with PLCβ to increase cAMP. Specific Gα donors for Gβγ subunits have previously been observed in certain cell types. For example, GIRK channels are activated by Gβγ subunits that are exclusively donated by $G\alpha_{i2}$ and $G\alpha_{i3}$ in native tissues, though any Gα subunit can donate the Gβγ 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 Gα and Gβγ subunits (Frank et al., 2005). Although Gα donor specificity has never to our knowledge been observed for Gβγ regulation of PLCβ, 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.

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 *Am*Dop3, 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 *Am*Dop3 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 $D_{2\alpha Pan}$ receptor will affect cAMP levels in pyloric neurons; though the data suggest that the $D_{2\alpha Pan}$ receptor will most likely couple with Gi and Go proteins to alter cAMP levels in pyloric neurons.

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 Gα subunits, and PLCβ, via Gβγ 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 $D_{1\alpha Pan}$ receptor couples with Gs and the $D_{1\beta Pan}$ receptor couples with both Gs and Gz when expressed in HEK cells (Clark and Baro, 2006). Likewise, we have shown that the $5\text{-}HT_{2\beta Pan}$ receptor couples with Gq (Clark et al., 2004) and the $5\text{-}HT_{1\alpha Pan}$ 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.

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 $D_{2\alpha Pan}$ receptor is heterologously expressed, it couples with Gi/o proteins and can modulate cAMP levels through both Gα and Gβγ subunits, like all of its homologs. The data also suggest that $D_{2\alpha Pan}$ 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.

Acknowledgements

We thank Nadja Spitzer and Elizabeth Prince for useful comments on the manuscript. We are grateful to Dr. Allison Mercer, University of Otago, for providing the *Am*Dop3 construct and to Dr. Robert Lefkowitz, Howard Hughes Medical Institute, for providing the $\beta ARK_{495–689}$. This work was supported, in part, by NIH NS38770 to DJB. MCC is a fellow of the Molecular Basis of Disease Program at Georgia State University.

References

Ahn S, Shenoy SK, Wei H, Lefkowitz RJ. Differential kinetic and spatial patterns of beta-arrestin and G protein-mediated ERK activation by the angiotensin II receptor. J Biol Chem 2004;279:35518–35525. [PubMed: 15205453]

Baillie GS, Sood A, McPhee I, Gall I, Perry SJ, Lefkowitz RJ, Houslay MD. beta-Arrestin-mediated PDE4 cAMP phosphodiesterase recruitment regulates beta-adrenoceptor switching from Gs to Gi. Proc Natl Acad Sci U S A 2003;100:940–945. [PubMed: 12552097]

Balfanz S, Strunker T, Frings S, Baumann A. A family of octopamine [corrected] receptors that specifically induce cyclic AMP production or Ca2+ release in Drosophila melanogaster. J Neurochem 2005;93:440–451. [PubMed: 15816867]

Banihashemi B, Albert PR. Dopamine-D2S receptor inhibition of calcium influx, adenylyl cyclase, and mitogen-activated protein kinase in pituitary cells: distinct Galpha and Gbetagamma requirements. Mol Endocrinol 2002;16:2393–2404. [PubMed: 12351703]

Beaulieu JM, Sotnikova TD, Marion S, Lefkowitz RJ, Gainetdinov RR, Caron MG. An Akt/beta-arrestin 2/PP2A signaling complex mediates dopaminergic neurotransmission and behavior. Cell 2005;122:261–273. [PubMed: 16051150]

Beggs KT, Hamilton IS, Kurshan PT, Mustard JA, Mercer AR. Characterization of a D2-like dopamine receptor (AmDOP3) in honey bee, Apis mellifera. Insect Biochem Mol Biol 2005;35:873–882. [PubMed: 15944083]

Blackmer T, Larsen EC, Bartleson C, Kowalchyk JA, Yoon EJ, Preininger AM, Alford S, Hamm HE, Martin TF. G protein betagamma directly regulates SNARE protein fusion machinery for secretory granule exocytosis. Nat Neurosci 2005;8:421–425. [PubMed: 15778713]

Blenau W, Baumann A. Molecular and pharmacological properties of insect biogenic amine receptors: lessons from Drosophila melanogaster and Apis mellifera. Arch Insect Biochem Physiol 2001;48:13–38. [PubMed: 11519073]

Blenau W, Erber J, Baumann A. Characterization of a dopamine D1 receptor from Apis mellifera: cloning, functional expression, pharmacology, and mRNA localization in the brain. J Neurochem 1998;70:15–23. [PubMed: 9422342]

Cabrera-Vera TM, Vanhauwe J, Thomas TO, Medkova M, Preininger A, Mazzoni MR, Hamm HE. Insights into G protein structure, function, and regulation. Endocr Rev 2003;24:765–781. [PubMed: 14671004]

Cazzamali G, Klaerke DA, Grimmelikhuijzen CJ. A new family of insect tyramine receptors. Biochem Biophys Res Commun 2005;338:1189–1196. [PubMed: 16274665]

Clark MC, Baro DJ. Molecular cloning and characterization of crustacean type-one dopamine receptors: D(1alphaPan) and D(1betaPan). Comp Biochem Physiol B Biochem Mol Biol 2006;143:294–301. [PubMed: 16426885]

Clark MC, Dever TE, Dever JJ, Xu P, Rehder V, Sosa MA, Baro DJ. Arthropod 5-HT2 receptors: a neurohormonal receptor in decapod crustaceans that displays agonist independent activity resulting from an evolutionary alteration to the DRY motif. J Neurosci 2004;24:3421–3435. [PubMed: 15056722]

Cotecchia S, Exum S, Caron MG, Lefkowitz RJ. Regions of the alpha 1-adrenergic receptor involved in coupling to phosphatidylinositol hydrolysis and enhanced sensitivity of biological function. Proc Natl Acad Sci U S A 1990;87:2896–2900. [PubMed: 2158097]

Daaka Y, Luttrell LM, Lefkowitz RJ. Switching of the coupling of the beta2-adrenergic receptor to different G proteins by protein kinase A. Nature 1997;390:88–91. [PubMed: 9363896]

Dascal N. Ion-channel regulation by G proteins. Trends Endocrinol Metab 2001;12:391–398. [PubMed: 11595540]

Evans PD, Maqueira B. Insect octopamine receptors: a new classification scheme based on studies of cloned Drosophila G-protein coupled receptors. Invert Neurosci 2005;5:111–118. [PubMed: 16211376]

Federman AD, Conklin BR, Schrader KA, Reed RR, Bourne HR. Hormonal stimulation of adenylyl cyclase through Gi-protein beta gamma subunits. Nature 1992;356:159–161. [PubMed: 1312225]

Feng G, Hannan F, Reale V, Hon YY, Kousky CT, Evans PD, Hall LM. Cloning and functional characterization of a novel dopamine receptor from Drosophila melanogaster. J Neurosci 1996;16:3925–3933. [PubMed: 8656286]

Flamm RE, Harris-Warrick RM. Aminergic modulation in lobster stomatogastric ganglion. II. Target neurons of dopamine, octopamine, and serotonin within the pyloric circuit. J Neurophysiol 1986;55:866–881. [PubMed: 3086514]

Frank M, Thumer L, Lohse MJ, Bunemann M. G Protein activation without subunit dissociation depends on a G{alpha}(i)-specific region. J Biol Chem 2005;280:24584–24590. [PubMed: 15866880]

Franke RR, Konig B, Sakmar TP, Khorana HG, Hofmann KP. Rhodopsin mutants that bind but fail to activate transducin. Science 1990;250:123–125. [PubMed: 2218504]

Friedman J, Babu B, Clark RB. Beta(2)-adrenergic receptor lacking the cyclic AMP-dependent protein kinase consensus sites fully activates extracellular signal-regulated kinase 1/2 in human embryonic kidney 293 cells: lack of evidence for G(s)/G(i) switching. Mol Pharmacol 2002;62:1094–1102. [PubMed: 12391272]

Gerachshenko T, Blackmer T, Yoon EJ, Bartleson C, Hamm HE, Alford S. Gbetagamma acts at the C terminus of SNAP-25 to mediate presynaptic inhibition. Nat Neurosci 2005;8:597–605. [PubMed: 15834421]

Ghahremani MH, Cheng P, Lembo PM, Albert PR. Distinct roles for Galphai2, Galphai3, and Gbeta gamma in modulation of forskolin- or Gs-mediated cAMP accumulation and calcium mobilization by dopamine D2S receptors. J Biol Chem 1999;274:9238–9245. [PubMed: 10092597]

Gotzes F, Balfanz S, Baumann A. Primary structure and functional characterization of a Drosophila dopamine receptor with high homology to human D1/5 receptors. Receptors Channels 1994;2:131–141. [PubMed: 7953290]

Gruhn M, Guckenheimer J, Land B, Harris-Warrick RM. Dopamine modulation of two delayed rectifier potassium currents in a small neural network. J Neurophysiol 2005;94:2888–2900. [PubMed: 16014791]

Han KA, Millar NS, Grotewiel MS, Davis RL. DAMB, a novel dopamine receptor expressed specifically in Drosophila mushroom bodies. Neuron 1996;16:1127–1135. [PubMed: 8663989]

Harris-Warrick; Marder, E. Dynamic Biological Networks: the stomatogastric nervous system. Cambridge: MIT Press; 1992.

Harris-Warrick RM, Coniglio LM, Barazangi N, Guckenheimer J, Gueron S. Dopamine modulation of transient potassium current evokes phase shifts in a central pattern generator network. J Neurosci 1995a;15:342–358. [PubMed: 7823140]

Harris-Warrick RM, Coniglio LM, Levini RM, Gueron S, Guckenheimer J. Dopamine modulation of two subthreshold currents produces phase shifts in activity of an identified motoneuron. J Neurophysiol 1995b;74:1404–1420. [PubMed: 8989381]

Harris-Warrick RM, Johnson BR, Peck JH, Kloppenburg P, Ayali A, Skarbinski J. Distributed effects of dopamine modulation in the crustacean pyloric network. Ann N Y Acad Sci 1998;860:155–167. [PubMed: 9928309]

Hearn MG, Ren Y, McBride EW, Reveillaud I, Beinborn M, Kopin AS. A Drosophila dopamine 2-like receptor: Molecular characterization and identification of multiple alternatively spliced variants. Proc Natl Acad Sci U S A 2002;99:14554–14559. [PubMed: 12391323]

Hernandez-Lopez S, Tkatch T, Perez-Garci E, Galarraga E, Bargas J, Hamm H, Surmeier DJ. D2 dopamine receptors in striatal medium spiny neurons reduce L-type Ca2+ currents and excitability via a novel PLC[beta]1-IP3-calcineurin-signaling cascade. J Neurosci 2000;20:8987–8995. [PubMed: 11124974]

Ivanina T, Varon D, Peleg S, Rishal I, Porozov Y, Dessauer CW, Keren-Raifman T, Dascal N. Galphai1 and Galphai3 differentially interact with, and regulate, the G protein-activated K+ channel. J Biol Chem 2004;279:17260–17268. [PubMed: 14963032]

Johnson BR, Kloppenburg P, Harris-Warrick RM. Dopamine modulation of calcium currents in pyloric neurons of the lobster stomatogastric ganglion. J Neurophysiol 2003;90:631–643. [PubMed: 12904487]

Kimura K, Sela S, Bouvier C, Grandy DK, Sidhu A. Differential coupling of D1 and D5 dopamine receptors to guanine nucleotide binding proteins in transfected GH4C1 rat somatomammotrophic cells. J Neurochem 1995a;64:2118–2124. [PubMed: 7722495]

Kimura K, White BH, Sidhu A. Coupling of human D-1 dopamine receptors to different guanine nucleotide binding proteins. Evidence that D-1 dopamine receptors can couple to both Gs and G(o). J Biol Chem 1995b;270:14672–14678. [PubMed: 7782330]

Kloppenburg P, Levini RM, Harris-Warrick RM. Dopamine modulates two potassium currents and inhibits the intrinsic firing properties of an identified motor neuron in a central pattern generator network. J Neurophysiol 1999;81:29–38. [PubMed: 9914264]

Kloppenburg P, Zipfel WR, Webb WW, Harris-Warrick RM. Highly localized Ca(2+) accumulation revealed by multiphoton microscopy in an identified motoneuron and its modulation by dopamine. J Neurosci 2000;20:2523–2533. [PubMed: 10729332]

Koch WJ, Hawes BE, Inglese J, Luttrell LM, Lefkowitz RJ. Cellular expression of the carboxyl terminus of a G protein-coupled receptor kinase attenuates G beta gamma-mediated signaling. J Biol Chem 1994;269:6193–6197. [PubMed: 8119963]

Lee FJ, Liu F. Direct interactions between NMDA and D1 receptors: a tale of tails. Biochem Soc Trans 2004;32:1032–1036. [PubMed: 15506956]

Lefkowitz RJ, Pierce KL, Luttrell LM. Dancing with different partners: protein kinase a phosphorylation of seven membrane-spanning receptors regulates their G protein-coupling specificity. Mol Pharmacol 2002;62:971–974. [PubMed: 12391258]

Lefkowitz RJ, Shenoy SK. Transduction of receptor signals by beta-arrestins. Science 2005;308:512–517. [PubMed: 15845844]

Lezcano N, Mrzljak L, Eubanks S, Levenson R, Goldman-Rakic P, Bergson C. Dual signaling regulated by calcyon, a D1 dopamine receptor interacting protein. Science 2000;287:1660–1664. [PubMed: 10698743]

Limbird LE. The receptor concept: a continuing evolution. Mol Interv 2004;4:326–336. [PubMed: 15616162]

Liu F, Wan Q, Pristupa ZB, Yu XM, Wang YT, Niznik HB. Direct protein-protein coupling enables cross-talk between dopamine D5 and gamma-aminobutyric acid A receptors. Nature 2000;403:274–280. [PubMed: 10659839]

Maqueira B, Chatwin H, Evans PD. Identification and characterization of a novel family of Drosophila beta-adrenergic-like octopamine G-protein coupled receptors. J Neurochem 2005;94:547–560. [PubMed: 15998303]

Marder E, Bucher D. Central pattern generators and the control of rhythmic movements. Curr Biol 2001;11:R986–R996. [PubMed: 11728329]

Missale C, Nash SR, Robinson SW, Jaber M, Caron MG. Dopamine receptors: from structure to function. Physiol Rev 1998;78:189–225. [PubMed: 9457173]

Mustard JA, Blenau W, Hamilton IS, Ward VK, Ebert PR, Mercer AR. Analysis of two D1-like dopamine receptors from the honey bee Apis mellifera reveals agonist-independent activity. Brain Res Mol Brain Res 2003;113:67–77. [PubMed: 12750008]

Neve KA, Seamans JK, Trantham-Davidson H. Dopamine receptor signaling. J Recept Signal Transduct Res 2004;24:165–205. [PubMed: 15521361]

Nguyen CH, Watts VJ. Dexras1 blocks receptor-mediated heterologous sensitization of adenylyl cyclase 1. Biochem Biophys Res Commun 2005;332:913–920. [PubMed: 15913563]

Nusbaum MP, Beenhakker MP. A small-systems approach to motor pattern generation. Nature 2002;417:343–350. [PubMed: 12015615]

O'Sullivan GJ, Roth BL, Kinsella A, Waddington JL. SK&F 83822 distinguishes adenylyl cyclase from phospholipase C-coupled dopamine D1-like receptors: behavioural topography. Eur J Pharmacol 2004;486:273–280. [PubMed: 14985049]

Oakley RH, Laporte SA, Holt JA, Barak LS, Caron MG. Association of beta-arrestin with G protein-coupled receptors during clathrin-mediated endocytosis dictates the profile of receptor resensitization. J Biol Chem 1999;274:32248–32257. [PubMed: 10542263]

Obadiah J, Avidor-Reiss T, Fishburn CS, Carmon S, Bayewitch M, Vogel Z, Fuchs S, Levavi-Sivan B. Adenylyl cyclase interaction with the D2 dopamine receptor family; differential coupling to Gi, Gz, and Gs. Cell Mol Neurobiol 1999;19:653–664. [PubMed: 10384262]

Peck JH, Nakanishi ST, Yaple R, Harris-Warrick RM. Amine modulation of the transient potassium current in identified cells of the lobster stomatogastric ganglion. J Neurophysiol 2001;86:2957–2965. [PubMed: 11731552]

Pei L, Lee FJ, Moszczynska A, Vukusic B, Liu F. Regulation of dopamine D1 receptor function by physical interaction with the NMDA receptors. J Neurosci 2004;24:1149–1158. [PubMed: 14762133]

Ramirez JM, Tryba AK, Pena F. Pacemaker neurons and neuronal networks: an integrative view. Curr Opin Neurobiol 2004;14:665–674. [PubMed: 15582367]

Rich TC, Fagan KA, Tse TE, Schaack J, Cooper DM, Karpen JW. A uniform extracellular stimulus triggers distinct cAMP signals in different compartments of a simple cell. Proc Natl Acad Sci USA 2001;98:13049–13054. [PubMed: 11606735]

Roeder T. Metabotropic histamine receptors--nothing for invertebrates? Eur J Pharmacol 2003;466:85–90. [PubMed: 12679144]

Sidhu A, Kimura K, Uh M, White BH, Patel S. Multiple coupling of human D5 dopamine receptors to guanine nucleotide binding proteins Gs and Gz. J Neurochem 1998;70:2459–2467. [PubMed: 9603210]

Srivastava DP, Yu EJ, Kennedy K, Chatwin H, Reale V, Hamon M, Smith T, Evans PD. Rapid, nongenomic responses to ecdysteroids and catecholamines mediated by a novel Drosophila G-protein-coupled receptor. J Neurosci 2005;25:6145–6155. [PubMed: 15987944]

Stehno-Bittel L, Krapivinsky G, Krapivinsky L, Perez-Terzic C, Clapham DE. The G protein beta gamma subunit transduces the muscarinic receptor signal for Ca2+ release in Xenopus oocytes. J Biol Chem 1995;270:30068–30074. [PubMed: 8530411]

Sullivan J. Finding the G spot on fusion machinery. Nat Neurosci 2005;8:542–544. [PubMed: 15856055]

Tierney AJ. Structure and function of invertebrate 5-HT receptors: a review. Comp Biochem Physiol A 2001;128:791–804.

Tsu RC, Wong YH. Gi-mediated stimulation of type II adenylyl cyclase is augmented by Gq-coupled receptor activation and phorbol ester treatment. J Neurosci 1996;16:1317–1323. [PubMed: 8778283]

Vortherms TA, Nguyen CH, Berlot CH, Watts VJ. Using molecular tools to dissect the role of Galphas in sensitization of AC1. Mol Pharmacol 2004;66:1617–1624. [PubMed: 15361543]

Watts VJ. Molecular mechanisms for heterologous sensitization of adenylate cyclase. J Pharmacol Exp Ther 2002;302:1–7. [PubMed: 12065693]

Watts VJ, Neve KA. Sensitization of adenylate cyclase by Galpha i/o-coupled receptors. Pharmacol Ther 2005;106:405–421. [PubMed: 15922020]

Werry TD, Sexton PM, Christopoulos A. "Ins and outs" of seven-transmembrane receptor signalling to ERK. Trends Endocrinol Metab 2005;16:26–33. [PubMed: 15620546]

Wong SK. G protein selectivity is regulated by multiple intracellular regions of GPCRs. Neurosignals 2003;12:1–12. [PubMed: 12624524]

Zaccolo M, Pozzan T. Discrete microdomains with high concentration of cAMP in stimulated rat neonatal cardiac myocytes. Science 2002;295:1711–1715. [PubMed: 11872839]

Zhen X, Goswami S, Abdali SA, Gil M, Bakshi K, Friedman E. Regulation of cyclin-dependent kinase 5 and calcium/calmodulin-dependent protein kinase II by phosphatidylinositol-linked dopamine receptor in rat brain. Mol Pharmacol 2004;66:1500–1507. [PubMed: 15286209]

Zheng S, Yu P, Zeng C, Wang Z, Yang Z, Andrews PM, Felder RA, Jose PA. Galpha12- and Galpha13-protein subunit linkage of D5 dopamine receptors in the nephron. Hypertension 2003;41:604–610. [PubMed: 12623966]

Zou S, Li L, Pei L, Vukusic B, Van Tol HH, Lee FJ, Wan Q, Liu F. Protein-protein coupling/uncoupling enables dopamine D2 receptor regulation of AMPA receptor-mediated excitotoxicity. J Neurosci 2005;25:4385–4395. [PubMed: 15858065]

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-OCH_3
: 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

Figure 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.

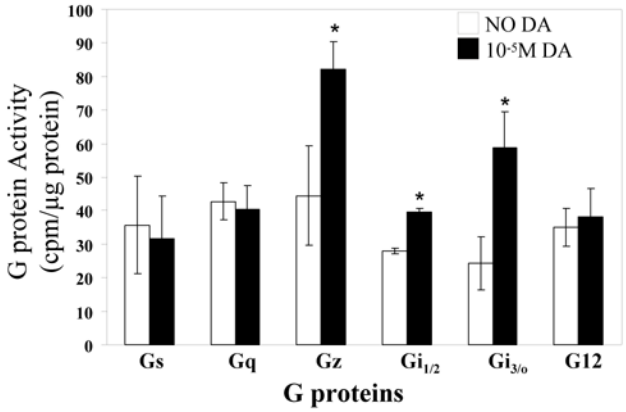

Figure 2. The $D_{2\alpha.1Pan}$ receptor couples with Gi/o family members

G protein activities in $HEKD_{2\alpha.1Pan}$ 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, Gi_1, Gi_2, Gi_3, Go, G12. Data represent the mean ± 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 ($p < 0.05$).

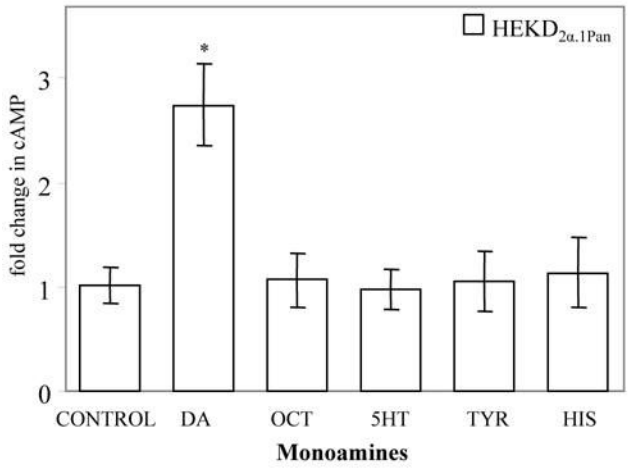

Figure 3. DA is the only monoamine that activates $D_{2\alpha.1Pan}$

Levels of cAMP (pmol cAMP/mg protein) were simultaneously measured in HEK and $HEKD_{2\alpha.1Pan}$ (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 $HEKD_{2\alpha.1Pan}$ 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, $n \geq 3$. The data were subjected to a one-way ANOVA.* indicates significant differences from cAMP levels measured in the control ($p < 0.05$).

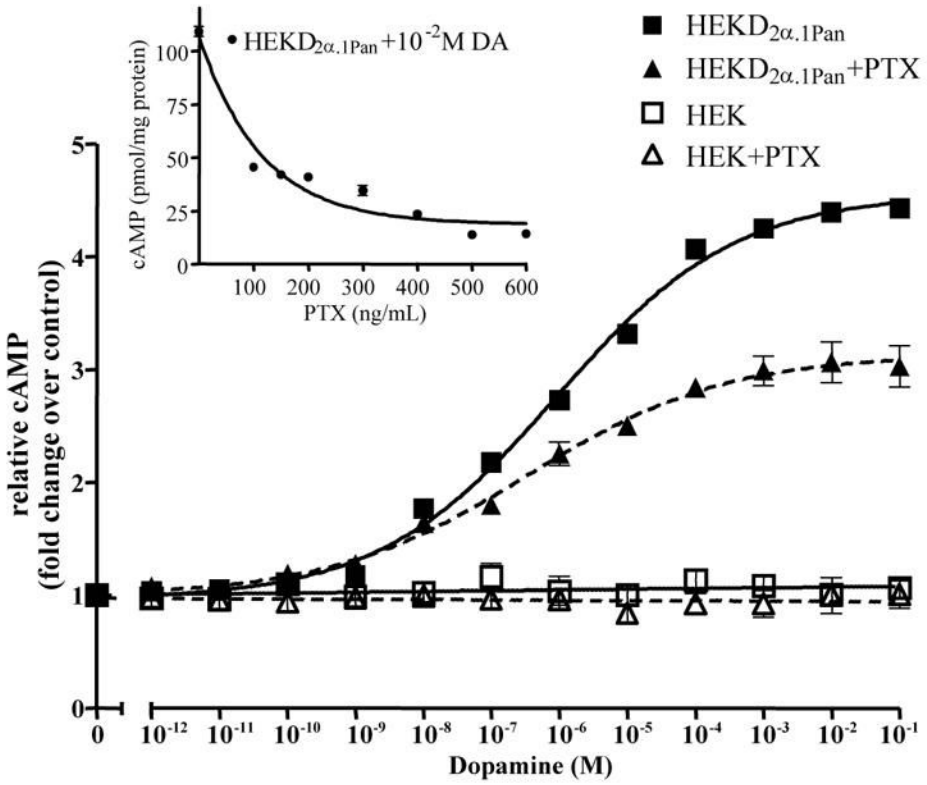

Figure 4. The $D_{2\alpha.1Pan}$ receptor couples positively with cAMP through PTX-sensitive Gi/o proteins, as well as PTX insensitive cascades

[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 (500ng/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 ± S.E.M.

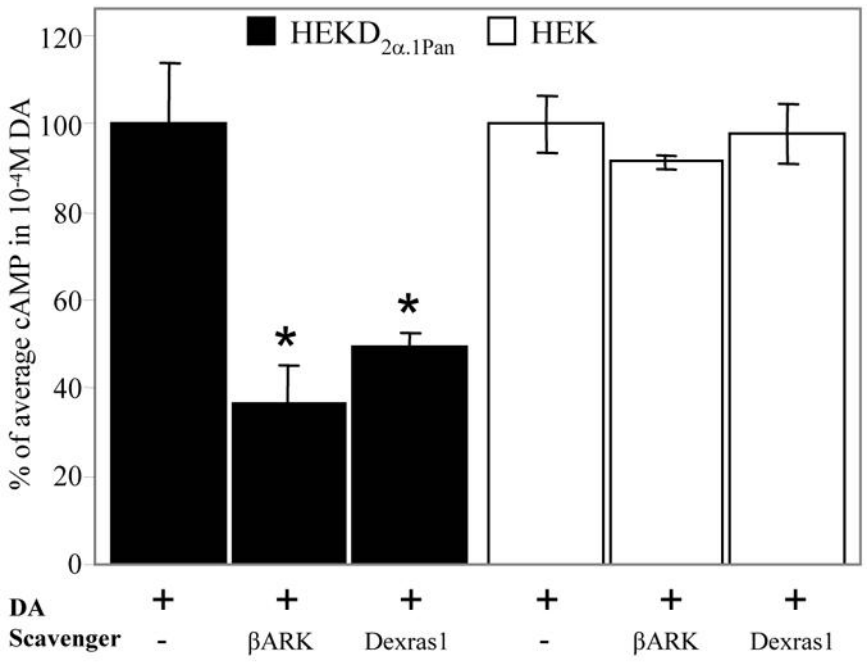

Figure 5. The increase in cAMP in HEKD$_{2\alpha.1Pan}$ is mediated by Gi/o βγ subunits

The Gβγ scavengers dexras1 or βARK$_{495–689}$ were transiently expressed in HEKD$_{2\alpha.1Pan}$ (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 dexras1or βARK$_{495–689}$ within a cell line ($p < 0.04$).

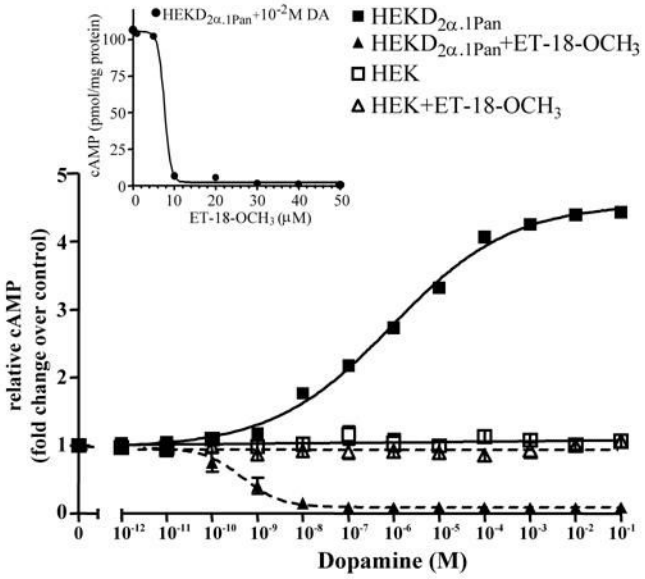

Figure 6. Blocking PLCβ reveals a negative coupling between the $D_{2\alpha.1Pan}$ receptor and cAMP

[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 PLCβ inhibitor, ET-18-OCH_3 (50μM). The inset represents an ET-18-OCH_3 dose-response curve for the HEK $D_{2\alpha.1Pan}$ cell line. All experiments were repeated 3 times. Data represent the mean ± S.E.M.

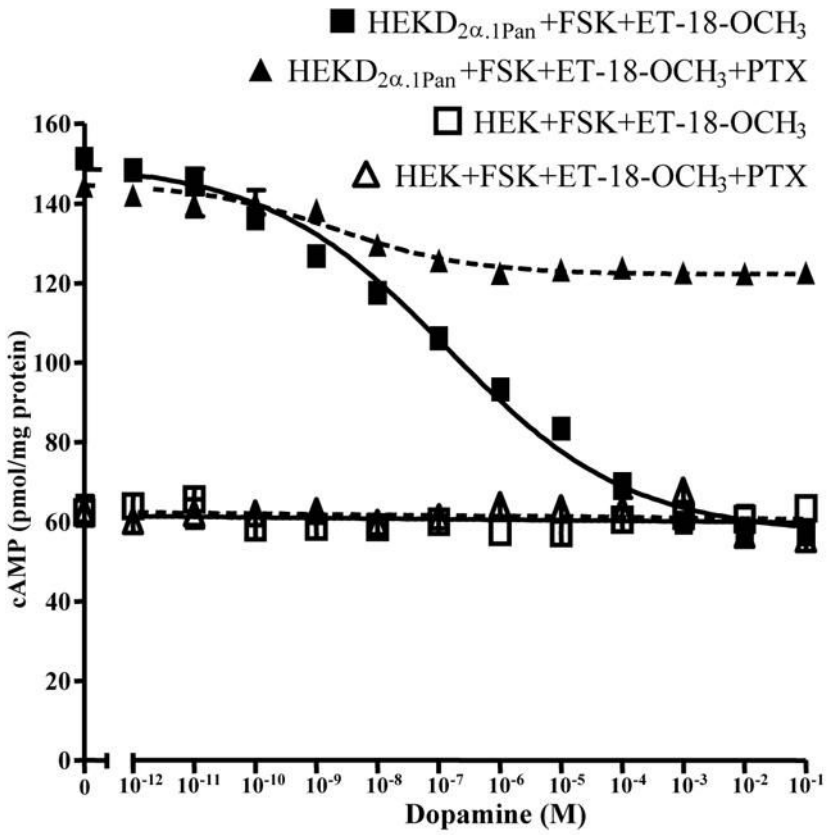

Figure 7. The negative coupling to cAMP is mediated by PTX-sensitive and PTX-insensitive G proteins

All cells were exposed to forskolin (2.5μM) and ET-18-OCH_3 (50 μM). [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. Experiments were conducted in the presence (dashed line) vs. the absence (solid line) of the Gi/o inhibitor, PTX (500ng/mL). All experiments were repeated 3 times. Data represent the mean ± S.E.M.

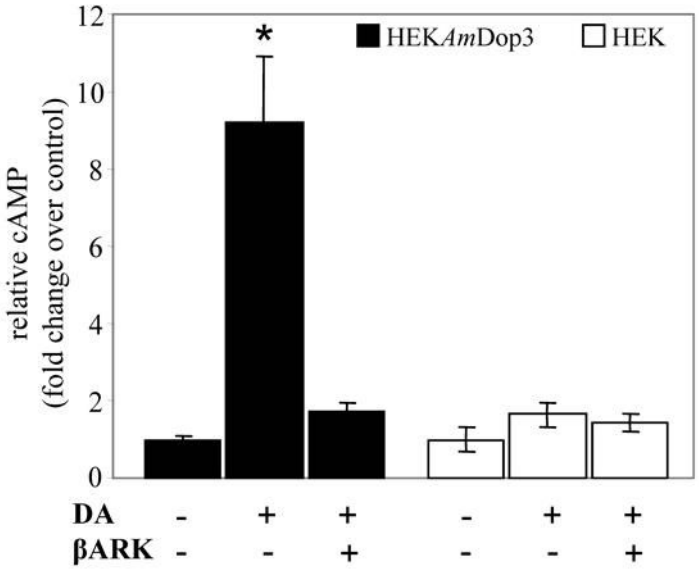

Figure 8. *Am*Dop3 positively couples to cAMP through the Gβγ cascade

Levels of cAMP (pmol cAMP/mg protein) were simultaneously measured in HEK cells expressing the *Am*Dop3 receptor (HEK*Am*Dop3, 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 Gβγ scavenger $\beta ARK_{495–689}$, 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 ± 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 ($p < 0.0001$).

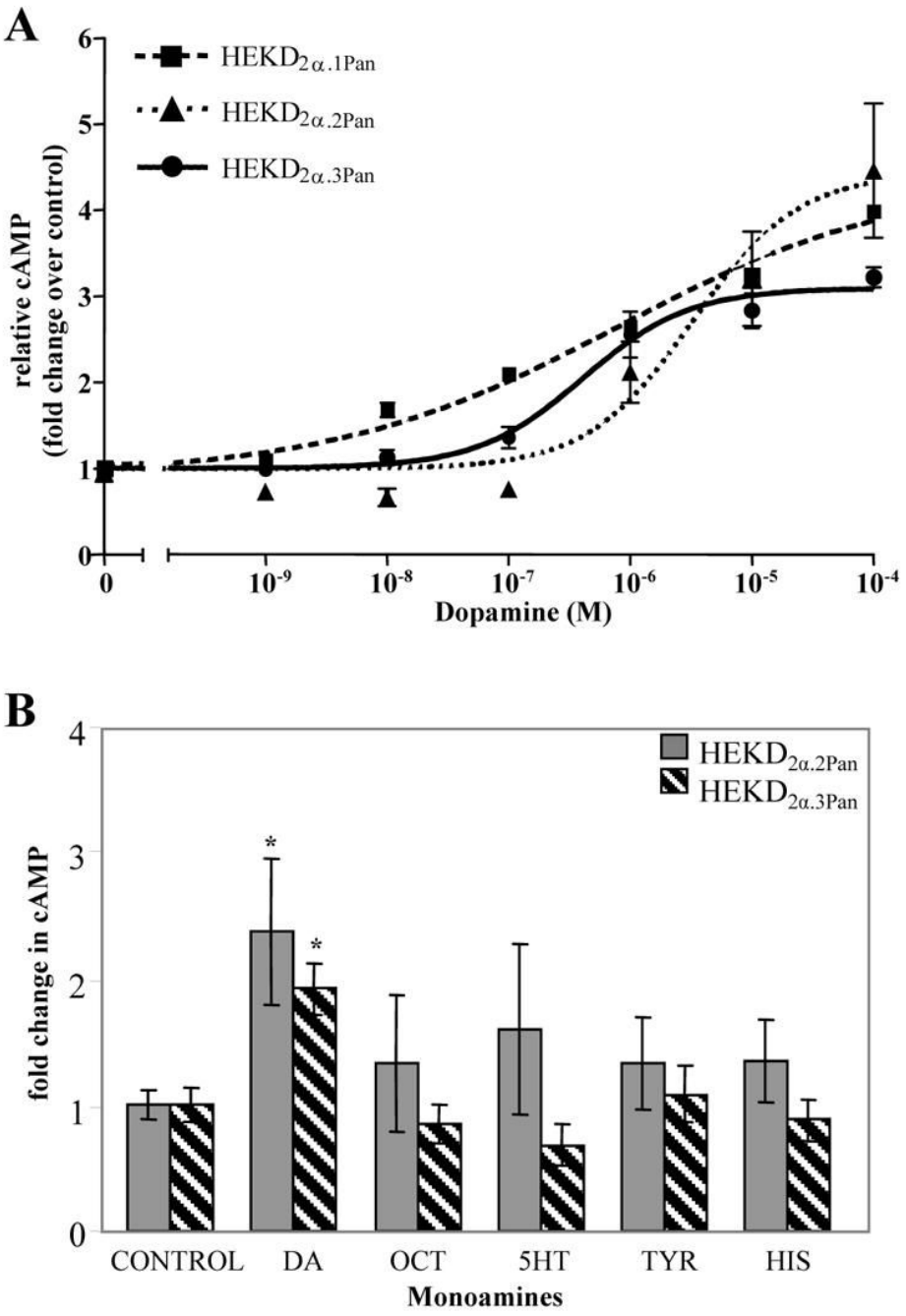

Figure 9. Alternate splicing changes the potency and efficacy of $D_{2\alpha Pan}$ isoforms

A. Changes in cAMP levels (pmol cAMP/mg protein) in response to increasing [DA] were measured in $HEKD_{2\alpha.1Pan}$ (squares, dashed line) $HEKD_{2\alpha.2Pan}$ (triangles, dotted line) and $HEKD_{2\alpha.3Pan}$ (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, $HEKD_{2\alpha.2Pan}$ (grey bars) and $HEKD_{2\alpha.3Pan}$ (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, $n \geq 3$. * indicates significant differences from cAMP levels measured in the control ($p < 0.05$).

Table 1

Alternate splicing of $D_{2\alpha Pan}$

Location	Isoform	Exon Configuration	Amino Acid Sequence,[1,2]
Intracellular loop 3	$D_{2\alpha Pan.1}$	+ i3 alternately spliced exon	...qdeeEEEGEDVMGLGGeenc...
	$D_{2\alpha Pan.2}$	+ i3 alternately spliced exon	...qdeeEEEGEDVMGLGGeenc...
	$D_{2\alpha Pan.3}$	− i3 alternately spliced exon	...qdeeeenc...
	$D_{2\alpha Pan.4}$	− i3 alternately spliced exon	...qdeeeenc...
C-terminus	$D_{2\alpha Pan.1}$	$D_{2\alpha}$ C-terminal exon 1	...ilisqS*
	$D_{2\alpha Pan.4}$	$D_{2\alpha}$ C-terminal exon 1	...ilisqS*
	$D_{2\alpha Pan.2}$	$D_{2\alpha}$ C-terminal exon 2	...ilisqMTISSNSFSLETVVLENHASC*
	$D_{2\alpha Pan.3}$	$D_{2\alpha}$ C-terminal exon 2	...ilisqMTISSNSFSLETVVLENHASC*

[1] The * represents a stop codon

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