

Evidence for Noncanonical Neurotransmitter Activation: Norepinephrine as a Dopamine D₂-Like Receptor Agonist[§]

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ABSTRACT

The G α i/o-coupled dopamine D₂-like receptor family comprises three subtypes: the D₂ receptor (D2R), with short and long isoform variants (D2SR and D2LR), D₃ receptor (D3R), and D₄ receptor (D4R), with several polymorphic variants. The common overlap of norepinephrine innervation and D₂-like receptor expression patterns prompts the question of a possible noncanonical action by norepinephrine. In fact, previous studies have suggested that norepinephrine can functionally interact with D4R. To our knowledge, significant interactions between norepinephrine and D2R or D3R receptors have not been demonstrated. By using radioligand binding and bioluminescent resonance energy transfer (BRET) assays in transfected cells, the present study attempted a careful comparison between dopamine and norepinephrine in their possible activation of all D₂-like receptors, including the two D2R isoforms and the most common D4R

polymorphic variants. Functional BRET assays included activation of G proteins with all G α i/o subunits, adenylyl cyclase inhibition, and β arrestin recruitment. Norepinephrine acted as a potent agonist for all D₂-like receptor subtypes, with the general rank order of potency of D3R > D4R \geq D2SR \geq D2L. However, for both dopamine and norepinephrine, differences depended on the G α i/o protein subunit involved. The most striking differences were observed with G α i2, where the rank order of potencies for both dopamine and norepinephrine were D4R > D2SR = D2LR \gg D3R. Furthermore the results do not support the existence of differences in the ability of dopamine and norepinephrine to activate different human D4R variants. The potency of norepinephrine for adrenergic α _{2A} receptor was only about 20-fold higher compared with D3R and D4R across the three functional assays.

Introduction

As with any other G protein-coupled receptors (GPCR), by classic radioligand binding studies, monoaminergic neurotransmitter receptors have been defined into subclasses of receptors with the name of the neurotransmitter with the highest affinity. Dopamine receptors are classified in two families, the G α s/olf-coupled D₁-like receptors, comprising D₁ and D₅ receptors (D1R and D5R) and the G α i/o-coupled D₂-like receptors, comprising D₂, D₃, and D₄ receptors (D2R, D3R, and D4R) (Beaulieu and Gainetdinov, 2011). D₂-like receptors are expressed in brain areas involved in neuropsychiatric disorders and constitute targets for neurologic and antipsychotic

medications. Structurally similar, the catecholamine norepinephrine is a metabolite of dopamine that requires dopamine β monooxygenase for the conversion. Therefore, the presence of the enzyme by the cell type distinguishes norepinephrine from dopamine release sites.

In general, norepinephrine and dopamine innervation coincides with the distribution of adrenergic and dopaminergic receptors, respectively, but this afferent-receptor coincidence is not always the case in the brain. Particularly in rodents, the cerebral cortex receives dense and widespread noradrenergic innervation, whereas dopaminergic terminals are more concentrated in the prefrontal cortex (Descarries et al., 1987; Séguéla et al., 1990). However, dopamine receptors are expressed throughout the cortex, and their localization exceeds that of the dopaminergic terminals (Lidow et al., 1989; Richfield et al., 1989; Goldsmith and Joyce, 1994; Wędzony et al., 2000). It has therefore been suggested that dopamine is a coneurotransmitter in noradrenergic neurons (Devoto and Flore, 2006) or that there is a long-range volume

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ABBREVIATIONS: α _{2A}AR, α _{2A} adrenergic receptor; BRET, bioluminescence resonance energy transfer; CAMYEL, cAMP sensor with yellow fluorescence protein-Epac-luciferase; D2R, D₂ receptor; D2SR, D₂ receptor short isoform; D2LR, D₂ receptor long isoform; D3R, D₃ receptor; D4R, D₄ receptor; D4.2R, D4.4R, D4.7R, D₄ receptor variant with 2-, 4-, 7-tandem repeats; FACS, fluorescence-activated cell sorting; DMSO, dimethylsulfoxide; GPCR, G protein-coupled receptor; GRK2, G protein-coupled receptor kinase 2; HEK, human embryonic kidney; L745,870, 3-[[4-(4-chlorophenyl)piperazin-1-yl]methyl]-1H-pyrrolo[2,3-b]pyridine; RLuc8, *Renilla* luciferase 8; 7-OH-DPAT, 7-hydroxy-N,N-di-n-propyl-2-aminotetralin; YFP, yellow fluorescence protein.

transmission of catecholamines (Rivera et al., 2008; Agnati et al., 2010). In fact, noradrenergic asynaptic varicosities constitute a majority of releasing sites in the cortex (Séguéla et al., 1990). Another mechanism, but specifically invoked for D4R (Lanau et al., 1997; Newman-Tancredi et al., 1997; Rivera et al., 2008; Cummings et al., 2010; Root et al., 2015), is the possibility that dopamine receptors can also be activated by endogenous norepinephrine.

Our current study investigates how norepinephrine compares with dopamine in ligand binding to effector-specific functional outcomes using bioluminescence resonance energy transfer (BRET) assays for all the D₂-like receptors. A canonical Gi/o noradrenergic receptor, the α_{2A} adrenergic receptor ($\alpha 2AR$), was also analyzed for comparison. This study included the short and long isoforms of D2R (D2SR and D2LR, respectively) and the three most common human polymorphic variants of the D4R gene, with 2-, 4-, or 7-nucleotide tandem repeats in the sequence encoding the third intracellular loop (D4.2R, D4.4R, and D4.7R). Thus, another unresolved issue about D₂-like receptors is the possible functional distinction among different isoform and polymorphic variants. For D2SR and D2LR, distinct presynaptic versus postsynaptic localization patterns respectively have been confirmed by various studies (Khan et al., 1998; Centonze et al., 2003; Lindgren et al., 2003), but there is no consensus about their main functional differences at the cellular level. Nevertheless, some studies indicate a differential dependence of D2SR and D2LR on Gi/o protein subunits for agonist-induced signaling (Gardner et al., 1996; Xu et al., 2002). Although initial studies seemed to indicate that the D4.7R signals with less efficiency than the most common variant D4.4R (Asghari et al., 1995; Jovanovic et al., 1999), more recent studies suggest that their differences might arise from specific protein interactions, including dopamine receptor heteromerization (González et al., 2012b; Ferré et al., 2014).

Materials and Methods

Radioligand Binding Experiments. Human embryonic kidney (HEK) 293 cells stably expressing human D2R, D3R, or D4.4R were grown in a 50:50 mix of Dulbecco's modified Eagle's medium and Ham's F-12 culture medium, supplemented with 20 mM HEPES, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1X antibiotic/antimycotic, 10% heat-inactivated fetal bovine serum, and 200 μ g/ml hygromycin (Life Technologies, Grand Island, NY) and kept in an incubator at 37°C and 5% CO₂. Upon reaching 80% to 90% confluence, cells were harvested using premixed Earle's Balanced Salt Solution with 5 μ M EDTA (Life Technologies) and centrifuged at 3000 rpm for 10 minutes at 21°C. The supernatant was removed, and the pellet was resuspended in 10 ml hypotonic lysis buffer (5 mM MgCl₂, 5 mM Tris, pH 7.4 at 4°C) and centrifuged at 20,000 rpm for 30 minutes at 4°C. The pellet was then resuspended in fresh binding buffer (50 mM Tris, 10 mM MgCl₂, 1 mM EDTA, pH 7.4). A Bradford protein assay (Bio-Rad Laboratories, Hercules, CA) was used to determine the protein concentration.

All test compounds were freshly dissolved in 30% dimethylsulfoxide (DMSO) and 70% H₂O to a stock concentration of 1 mM or 100 μ M. Each test compound was then diluted into 11 half-log serial dilutions using 30% DMSO vehicle; final test concentrations ranged from 10 μ M to 10 pM in duplicates. Competitive-inhibition experiments were conducted in 96-well plates containing 300 μ l fresh binding buffer, 50 μ l of diluted test compound, 100 μ l of membranes suspension (80 μ g/well for D2R, 40 μ g/well for D3R and 60 μ g/well for D4.4R), and 50 μ l of radioligand diluted in binding buffer (7-hydroxy-N,N-di-

propyl-2-aminotetralin: [³H]7-OH-DPAT; 1.5 nM final concentration for D2R, 0.5 nM final concentration for D3R, 3 nM final concentration for D4.4R; ARC, Saint Louis, MO). Nonspecific binding was determined using 10 μ M (+)-butaclamol (Sigma-Aldrich, St. Louis, MO), and total binding was determined with 30% DMSO vehicle.

The reaction was incubated for 90 minutes at room temperature and terminated by filtration through Uni-Filter-96 GF/B (PerkinElmer, Waltham, MA), presoaked in 0.5% polyethylenimine, using a Brandel 96-Well Plates Harvester Manifold (Brandel Instruments, Gaithersburg, MD). Filters were washed 3 times with 3 ml of ice-cold binding buffer; 65 μ l PerkinElmer MicroScint 20 Scintillation Cocktail was added, and the filters were counted using a PerkinElmer MicroBeta Microplate Counter. The IC₅₀ values for each compound were determined from dose-response curves, and the K_i values were calculated using the Cheng-Prusoff equation in GraphPad Prism 5 (GraphPad Software, San Diego, CA). Reported K_i values were determined from at least three independent experiments.

DNA Constructs and Transfection. For all the receptor constructs, a signal peptide followed by a Flag epitope tag were fused to the N terminus for enhanced cell surface expression (Guan et al., 1992) and their detection (Guo et al., 2008). Human receptor constructs were used for D2SR, D2LR, D3R, D4.2R, D4.4R, and D4.7R (Guitart et al., 2014; Frederick et al., 2015). For fusion receptor constructs with *Renilla* luciferase 8 (RLuc8; provided by Dr. S. Gambhir, Stanford University, Stanford, CA), the cDNA encoding full-length RLuc8 was fused in frame to the C terminus of receptors as reported elsewhere (Urizar et al., 2011). The following human G protein constructs were used: *Gai1*-mVenus with mVenus inserted at position 91, *Gai1*-, *Gai2*-, *Gai3*-, *Gao1*-, or *Gao2*-RLuc8 with RLuc8 inserted at position 91, *Gas*-RLuc8 with RLuc8 inserted at position 67, *Gaq*-RLuc8 with RLuc8 inserted at position 97, untagged *Gβ1*, untagged *Gγ2*, and *Gγ2* fused to full-length mVenus at its N terminus. The *Gα*-RLuc8 constructs were kindly provided by Dr. Céline Galés (INSERM, Toulouse, France). The cAMP sensor with yellow fluorescence protein (YFP)-Epac-RLuc (CAMYEL) was obtained from the American Type Culture Collection (no. MBA-277; ATCC, Manassas, VA) (Jiang et al., 2007). G protein-coupled receptor kinase 2 (GRK2) and mVenus-fused β arrestin-2 (Vishnivetskiy et al., 2011) constructs were obtained from Dr. J. Javitch (Columbia University, New York, NY). All the constructs were confirmed by sequencing analysis.

A constant amount of plasmid cDNA (15 μ g) was transfected into HEK 293T cells using polyethylenimine (Sigma-Aldrich) in a 1 to 2 ratio in 10-cm dishes. Cells were maintained in culture with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and kept in an incubator at 37°C and 5% CO₂. The transfected amount and ratio among the receptor and heterotrimeric G proteins were tested for optimized dynamic range in drug-induced BRET. The experiments were performed approximately 48-hours after transfection.

BRET Assay. Variations of BRET were performed to detect receptor ligand-induced events for: 1) Gi/o protein activation, 2) adenylyl cyclase inhibition, and 3) β arrestin recruitment.

Gi/o protein activation assay uses RLuc-fused *Gai/o* protein subunit and mVenus-fused *Gγ2* protein for resonance energy transfer (RET) pair. Flag-tagged receptor and untagged *Gβ1* constructs were cotransfected.

The adenylyl cyclase inhibition assay uses CAMYEL biosensor construct that contains RLuc and YFP allowing detection of intracellular cAMP change (Jiang et al., 2007). To study *Gai* dependent inhibition activity, cells were prestimulated with 10 μ M forskolin (Sigma-Aldrich) in the presence of 10 μ M propranolol 10 minutes before sample reading to ensure isolation of response from D₂-like receptors or $\alpha 2AR$.

The β arrestin recruitment uses RLuc-fused receptor and mVenus-fused β arrestin-2 for the RET pair. GRK2 was cotransfected to assist an enhanced phosphorylation required for the β arrestin recruitment.

As reported previously (Urizar et al., 2011), cells were harvested, washed, and resuspended in phosphate-buffered saline). Approximately 200,000 cells/well were distributed in 96-well plates, and 5 μ M coelenterazine H

TABLE 1

Binding affinities of dopamine and norepinephrine for D2LR, D2SR, D3R, and D4.4R

Results are from competitive inhibition experiments of [³H]7-OH-DPAT versus dopamine or norepinephrine in HEK-293 cells stably expressing D2LR, D2SR, D3R, or D4.4R. The equilibrium inhibition constant (K_i ; nM) is shown for dopamine or norepinephrine. Values are the mean \pm S.E.M. of three to six independent experiments.

Receptor	K_i (nM)		NE/DA (K_i)
	Dopamine	Norepinephrine	
D2LR	16 \pm 4	62 \pm 17	3.9
D2SR	12.5 \pm 3.1	75 \pm 10	6
D3R	3 \pm 1 ^a	43 \pm 15	14.3
D4.4R	6.4 \pm 2.0	68 \pm 25	10.6

NE/DA, ratio between K_i values of norepinephrine and dopamine for each receptor.

^a $P < 0.05$, statistically significantly different compared with D2LR (one-way analysis of variance, followed by post hoc Newman-Keuls test).

(substrate for luciferase) was added to each well. One minute after addition of coelenterazine H, dopamine (Sigma-Aldrich) or L-(−)-norepinephrine (Sigma-Aldrich) was added to each well. Antagonists were added 15 minutes before the addition of agonist. The fluorescence of the acceptor was quantified (excitation at 500 nm and emission at 540 nm for 1-second recordings) in Mithras LB940 (Berthold Technologies, Bad Wildbad, Germany) to confirm the constant expression levels across experiments.

In parallel, the BRET signal from the same batch of cells was determined as the ratio of the light emitted by mVenus (510–540 nm) over that emitted by RLuc (485 nm). Results are calculated for the BRET change (BRET ratio for the corresponding drug minus BRET ratio in the absence of the drug). E_{max} values are expressed as the subtracted BRET change times 1000 and in the dose–response graphs are normalized as percentage of maximal value. Data and statistical analysis were performed with Prism 5 (GraphPad Software).

FACS Assay. Fluorescence-activated cell sorting (FACS) was performed to determine the surface expression of each receptor construct, as described elsewhere (Costagliola et al., 1998), with FACS Canto II (BD Biosciences, San Jose, CA). Briefly, a fraction of the same cells used for BRET (~500,000 cells) were harvested and incubated with anti-Flag monoclonal antibody (Sigma-Aldrich) in FACS buffer (phosphate-buffered saline with 1% bovine serum albumin and 0.1% sodium azide), washed, and incubated with the secondary anti-mouse antibody conjugated with Alexa Fluor 647 (Invitrogen, Carlsbad, CA).

Statistics. Differences in K_i or EC_{50} values of norepinephrine and dopamine between different receptors were statistically analyzed by one-way analysis of variance followed by Newman-Keuls post hoc test (P values are as indicated in figure legends).

Results

Norepinephrine Binds to All Dopamine D₂-Like Receptor Subtypes with High Affinity in Stably Transfected HEK-293 Cells. Radioligand competition experiments with the D₂-like receptor agonist [³H]7-OH-DPAT (Lévesque et al., 1992) were performed in HEK-293 cells stably transfected with D2SR, D2LR, D3R, or D4.4R, to compare the affinities of dopamine and norepinephrine for the D₂-like receptors. Competition experiments revealed the high affinity (low K_i values) of dopamine for all the D₂-like receptors, with a rank order of D3R > D4.4R \geq D2SR = D2LR (Table 1). Consistent with previous reports (Missale et al., 1998), dopamine showed a K_i value for D3R significantly lower (higher affinity) than for D2LR. Norepinephrine also showed high and similar affinity (around 50 nM) for the four D₂-like receptor subtypes, with only 4-fold (D2LR) to 15-fold (D3R) less affinity compared with dopamine (Table 1).

Norepinephrine Signals with Similar Potency through All Dopamine D₂-Like Receptor Subtypes in Transiently Transfected HEK-293T Cells. D₂-like receptors, by activating Gi/o proteins, inhibit adenylyl cyclase, therefore decreasing intracellular cAMP levels; consequently, the activation of cAMP targets, such as cyclic nucleotide gated channels, exchange protein directly activated by cAMP (Epac) and cAMP-dependent protein kinase or protein kinase A (Antoni, 2012). To find a functional correlate of the results obtained with radioligand binding experiments, we measured D₂-like receptor-mediated inhibition of adenylyl-cyclase by measuring cAMP levels in intact cells transiently transfected with D2LR, D2SR, D3R or the D4R variants D4.2R, D4.4R, or D4.7R, using cAMP sensor with CAMYEL, a kinase-dead Epac I–based cAMP BRET biosensor. The model takes advantage of the conformational changes in Epac that are induced upon its binding to cAMP.

The conformational change triggered by cAMP results in a decrease in BRET due to luciferase and YFP changing their relative orientation from each other. A decrease in cAMP levels is therefore observed as an increase in BRET (Fig. 1) (Jiang et al., 2007). HEK-293T cells are reported to endogenously express β adrenergic receptors (Atwood et al., 2011). Accordingly, norepinephrine in nontransfected cells stimulated Gs-mediated cAMP increase, which could be completely inhibited by the selective β adrenergic blocker propranolol (10 μ M; Supplemental Fig. 1). Therefore, propranolol was added throughout the cAMP detection experiments to determine transfected D₂-like receptor-mediated responses.

The assay showed a high sensitivity and provided EC_{50} values for dopamine and norepinephrine that were similar, albeit slightly lower, to the K_i values observed in radioligand binding experiments (Fig. 1, Table 2). The slightly higher potency, particularly of dopamine, in the adenylyl cyclase inhibition assay compared with the radioligand displacement assay may be due to an artifactual ceiling effect set by potentially low cellular expression of the biosensor in a certain population of transfected cells. Depending on the proportion of low-expressing cells, the response of the decrease in cAMP production, read as an increase in BRET, may be seen prematurely as fully efficacious, giving a low estimate of EC_{50} .

Same as the binding experiments, both dopamine and norepinephrine showed low EC_{50} values with D3R, which were significantly different compared with D2LR (Table 2). In comparison with the binding experiments, both for dopamine and norepinephrine the EC_{50} values were moderately higher for D2LR than D2SR, although not statistically different for norepinephrine (Table 2). For dopamine, the EC_{50} values with the three D4R isoforms were as low as with D3R, and no statistically significant differences were obtained between the EC_{50} values for dopamine and norepinephrine in cells transfected with D4.2R, D4.4R, or D4.7R. In terms of efficacy, the E_{max} values for both dopamine and norepinephrine were statistically significantly higher for D2SR and lower for D3R compared with D2L, and no differences were observed among the D4R variants (Table 2).

Norepinephrine Activates Most G Proteins with Different Gai/o Subunits via D₂-Like Receptors at Submicromolar Concentrations in Transiently Transfected HEK-293T Cells. Gai/o protein subunits are associated with the canonical inhibition of adenylyl cyclase and can

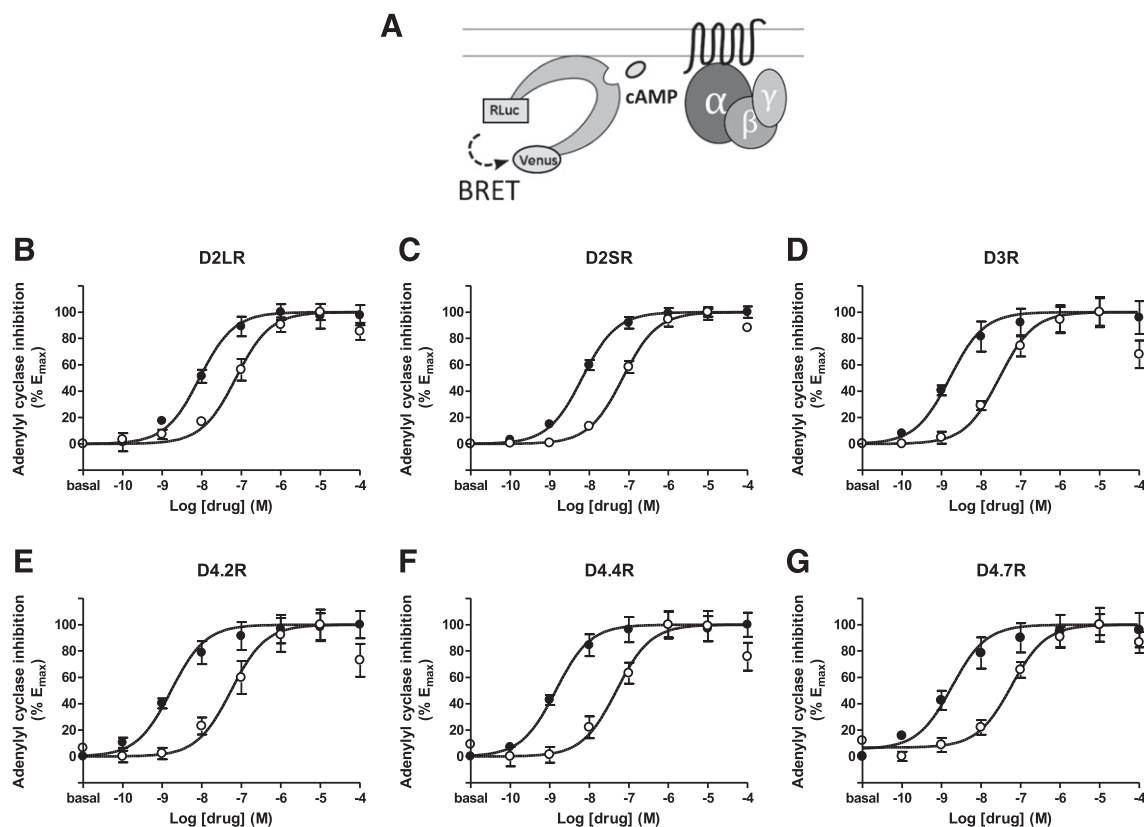


Fig. 1. (A) Scheme of the CAMYEL sensor configuration where the intramolecular conformational change triggered by cAMP results in a decrease in BRET due to RLuc8 and YFP changing their relative orientation from each other. (B–G) Dose–response experiments of adenylyl cyclase inhibition by dopamine (●) or norepinephrine (○) mediated by D2LR, D2SR, D3R, D4.2R, D4.4R, and D4.7R in HEK-293T cells transiently expressing the CAMYEL sensor and the indicated D₂-like receptor: (B) D2LR, (C) D2SR, (D) D3R, (E) D4.2R, (F) D4.4R, or (G) D4.7R. Cells were treated with forskolin (10 μ M) for 10 minutes followed by dopamine or norepinephrine and BRET values obtained by forskolin alone were subtracted from BRET values for each agonist concentration. Data represent the mean \pm S.E.M. of 6 to 11 experiments performed in triplicate and are shown as a percentage of the maximal effect (see Table 2 for EC_{50} and E_{max} values and statistical comparisons).

be grouped in *Gai*, comprising the *Gai1*, *Gai2*, and *Gai3* subunits, and *Gao*, comprising the *Gao1* and *Gao2* splice variants. Studies have suggested the existence of differences in the ability of GPCR to activate G proteins with different *Gai/o* subunits, including different D₂-like receptor subtypes (see *Discussion* and Montmayeur et al., 1993; Liu et al., 1994;

Lane et al., 2008), which coincides with different proportions of G protein subunits expressed in different cell types and brain regions (Orford et al., 1991; Jiang et al., 2001).

Therefore, using BRET methodology, we compared the ability of dopamine and norepinephrine to activate *Gi/o* proteins with different *Gai/o* subunits via all different D₂-like receptor subtypes, including the two D2R isoform variants and the three main D4R polymorphic variants. For this purpose, we used luciferase-fused *Gai1*, *Gai2*, *Gai3*, *Gao1*, or *Gao2* and Venus-fused γ 2 subunit constructs. β 1 and γ 2 were chosen because of their wide expression in the brain.

As characterized thoroughly in previous reports (Galés et al., 2005, 2006), the ligand-induced G protein conformational change results in negative BRET change, consistent with the opening and distancing between the *Gai* and γ 2 proteins, which is interpreted as the activation of the heterotrimeric G protein. Taking advantage of a high sequence homology among the five *Gai/o* protein subunits, constructs with a luciferase insertion in the same position (i.e., amino acid 91) were used. Luminescence and fluorescence were always measured to control for similar expression levels of transiently transfected G proteins (luciferase-*Gai*, β 1, and Venus- γ 2). Receptor constructs were fused to a Flag epitope at the N terminus, which allowed measurements of cell-surface expression by anti-Flag immunostaining by FACS.

To avoid a significant influence by spare receptors, an amount for the receptor cDNA that proportionately increased

TABLE 2

Adenylyl cyclase inhibition by dopamine and norepinephrine mediated by D2LR, D2SR, D3R, D4.2R, D4.4R, and D4.7R

Results are from adenylyl cyclase inhibition-BRET experiments in HEK-293T cells transiently transfected with the CAMYEL sensor and one of the indicated receptors. Data were fit by nonlinear regression to a sigmoidal dose–response relationship against the agonist concentration. The EC_{50} and E_{max} values are mean \pm S.E.M. of 5 to 11 experiments performed in triplicate. E_{max} values are expressed as $1000 \times [BRET_{max} - BRET_{basal}]$.

Receptor	Dopamine		Norepinephrine		NE/DA (EC_{50})
	EC_{50} (nM)	E_{max}	EC_{50} (nM)	E_{max}	
D2LR	9.4 \pm 1.1	94 \pm 8	88 \pm 17	94 \pm 6	9.4
D2SR	6.7 \pm 0.5 ^a	140 \pm 7 ^a	62 \pm 6	149 \pm 8 ^a	9.3
D3R	1.6 \pm 0.2 ^a	48 \pm 7 ^a	19 \pm 3 ^a	50 \pm 5 ^a	12
D4.2R	1.8 \pm 0.2 ^a	74 \pm 8	54 \pm 8	69 \pm 8	30
D4.4R	1.4 \pm 0.2 ^a	98 \pm 9	53 \pm 8	79 \pm 6	38
D4.7R	1.6 \pm 0.3 ^a	83 \pm 11	53 \pm 13	84 \pm 6	33

NE/DA, ratio between EC_{50} values of norepinephrine and dopamine for each receptor.

^a $P < 0.001$, significantly statistically different compared with D2LR (one-way analysis of variance, followed by post hoc Newman-Keuls test).

the surface receptor expression as well as E_{\max} was chosen for the study (data not shown). FACS analysis also showed no statistically significant differences in the cell surface expression among the D2R and D4R variants transfected (Supplemental Fig. 2).

The EC_{50} values for both dopamine and norepinephrine were in the submicromolar range for most D₂-like receptors and Gai/o protein subunits, and they were relatively higher than those obtained in radioligand displacement and adenylyl cyclase inhibition experiments (Fig. 2, Table 3). Gi/o protein coupling specificity was demonstrated by the absence of Gs or Gq protein activation by D₂-like receptors; the absence of dopamine- or norepinephrine-induced Gai1 protein activation via endogenously expressed receptors was confirmed in cells without transfected D₂-like receptors (Supplemental Fig. 3). In addition, selective involvement of transfected D₂-like receptors in dopamine and norepinephrine-induced G protein activation (with the Gai1 subunit) was demonstrated by dose-dependent blockade with the D4R antagonist L745,870 (3-[[4-(4-chlorophenyl)piperazin-1-yl]methyl]-1H-pyrrolo[2,3-b]pyridine) and the D2R-D3R antagonist raclopride (Supplemental Fig. 4).

Dopamine was more potent than norepinephrine for most D₂-like receptors and Gai/o protein subunits, but significant Gai/o protein subunit-dependent differences were observed (Table 3). For the two D2R and the three D4R variants, dopamine was around 10 times more potent than norepinephrine when coupling to Gai1, Gai2, or Gai3, but only around 2

times more potent when coupling to G α 1 or G α 2 (Table 3). Dopamine and norepinephrine showed higher potencies for the D4R than for the D2R variants when coupled to Gai1 and Gai2 proteins but similar potencies when coupled to G α 1 or G α 2. When coupling to Gai3, dopamine was more potent for D4R than for D2R, but norepinephrine showed similar potencies. Dopamine and norepinephrine showed higher potency for D3R than for the other D₂-like receptors with all Gai/o protein subunits with the exception of Gai2, which seemed to be completely insensitive to dopamine or norepinephrine-induced D3R activation.

No statistically significant differences between D4R variants were observed. On the other hand, a significantly higher potency of dopamine and norepinephrine could be observed at D2SR compared with D2LR with all Gai/o protein subunits but Gai2. The same as for the adenylyl cyclase signaling experiments, for most Gai/o protein subunits the E_{\max} values for both dopamine and norepinephrine were significantly higher for D2SR and lower for D3R compared with D2L, and no major differences were observed among the D4R variants (Table 3).

Norepinephrine Promotes β Arrestin-2 Recruitment to the D₂-Like Receptor in Transiently Transfected HEK-293T Cells. Receptor activation also leads to β arrestin-mediated receptor recruitment and its own signaling events (Shenoy and Lefkowitz, 2011), which are major effector-triggered events besides the G protein-mediated signal transduction. Hence, dopamine and norepinephrine-induced β arrestin recruitment across all the D₂-like receptors were also

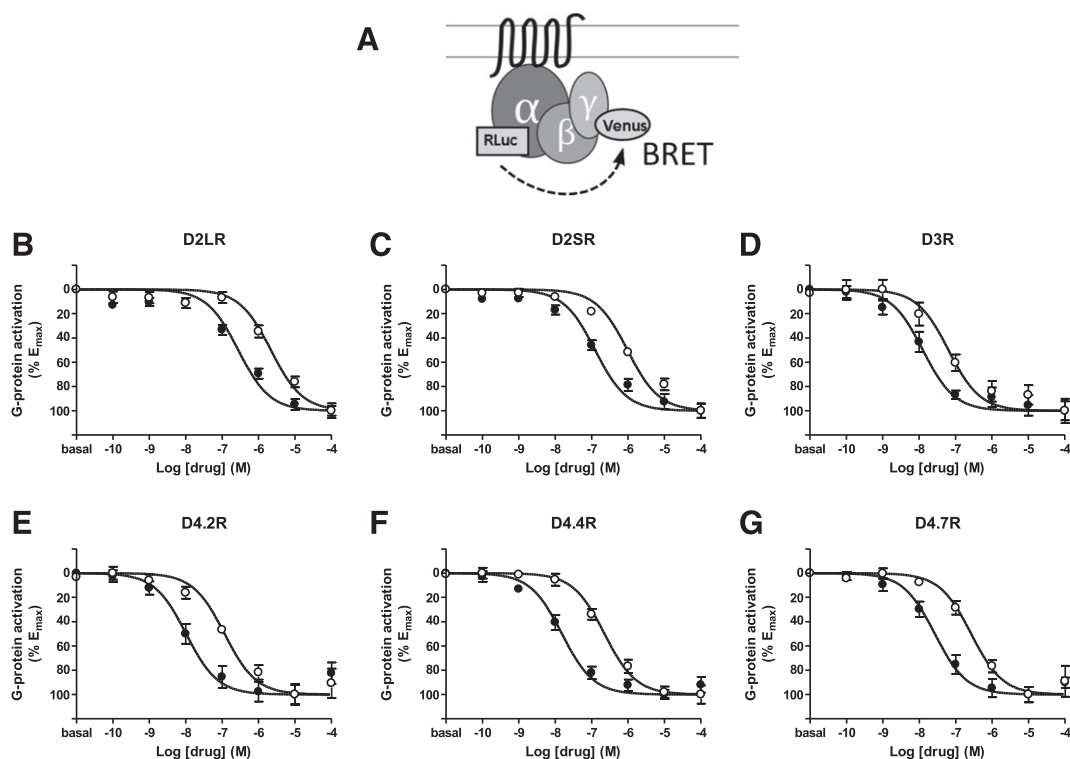


Fig. 2. (A) Scheme of the constructs used for G-protein activation BRET experiments where RLuc8 is fused to the Gai1 subunit and mVenus is fused to the γ_2 subunit. (B–G) Dose–response experiments of G protein activation by dopamine (●) or norepinephrine (○) mediated by D2LR, D2SR, D3R, D4.2R, D4.4R, and D4.7R in HEK-293T cells transiently expressing the G protein subunits Gai1-RLuc8, unfused β_1 and γ_2 -mVenus and the indicated D₂-like receptor: (B) D2LR, (C) D2SR, (D) D3R, (E) D4.2R, (F) D4.4R, or (G) D4.7R. Cells were treated with coelenterazine H followed by increasing concentrations of dopamine or norepinephrine. After 10 minutes, BRET between G-protein subunits was measured as described in *Materials and Methods*. BRET values in the absence of ligands were subtracted from the BRET values for each agonist concentration. Data were fit by nonlinear regression to a sigmoidal dose–response relationship against the agonist concentration and are shown as a percentage of the maximal effect. Data represent the mean \pm S.E.M. of 11 to 18 experiments performed in triplicate (see Table 3 for EC_{50} and E_{\max} values and statistical comparisons).

TABLE 3

G-protein activation by dopamine and norepinephrine mediated by D2LR, D2SR, D3R, D4.2R, D4.4R, and D4.7R involving different *Gai/o* subtypes

Results were obtained from G protein activation-BRET experiments in HEK-293T cells transiently transfected with different *Gai*-RLuc8 subtypes (*Gai1*, *Gai2*, *Gai3*, *Gao1*, *Gao2*) and γ_2 -mVenus in cells expressing different D₂-like receptors. Data were fit by nonlinear regression to a sigmoidal dose-response relationship against the agonist concentration. The EC₅₀ and *E*_{max} values are the mean \pm S.E.M. of 5 to 18 experiments performed in triplicate. *E*_{max} values are expressed as 1000 \times [BRET_{max} - BRET_{basal}].

G α Subunit	Receptor	Dopamine		Norepinephrine		NE/DA (EC ₅₀)
		EC ₅₀ (nM)	<i>E</i> _{max}	EC ₅₀ (nM)	<i>E</i> _{max}	
<i>Gai1</i>	D2LR	393 \pm 43	19 \pm 1	3609 \pm 549	17 \pm 1	9.2
	D2SR	161 \pm 24 ^a	30 \pm 1 ^a	987 \pm 171 ^a	26 \pm 1 ^a	6.1
	D3R	12 \pm 3 ^a	15 \pm 1	83 \pm 33 ^a	15 \pm 1	6.9
	D4.2R	18 \pm 5 ^a	17 \pm 1	116 \pm 28 ^a	16 \pm 2	6.4
	D4.4R	21 \pm 6 ^a	22 \pm 1	215 \pm 31 ^a	20 \pm 1	10.2
<i>Gai2</i>	D4.7R	26 \pm 5 ^a	25 \pm 2 ^b	229 \pm 44 ^a	19 \pm 1	8.8
	D2LR	177 \pm 46	21 \pm 1	1765 \pm 391	24 \pm 4	10
	D2SR	92 \pm 10 ^b	41 \pm 2 ^a	1280 \pm 309	39 \pm 2 ^c	14
	D3R	ND	ND	ND	ND	—
	D4.2R	24 \pm 6 ^a	37 \pm 3 ^a	340 \pm 69 ^c	31 \pm 5	14
<i>Gai3</i>	D4.4R	19 \pm 2 ^a	37 \pm 4 ^a	134 \pm 30 ^c	35 \pm 2 ^b	7
	D4.7R	28 \pm 8 ^c	45 \pm 3 ^a	285 \pm 46 ^c	47 \pm 3 ^a	10.2
	D2LR	132 \pm 34	26 \pm 2	1198 \pm 160	26 \pm 3	9.1
	D2SR	52 \pm 8 ^a	40 \pm 2 ^a	379 \pm 103 ^a	40 \pm 2 ^c	7.3
	D3R	3.6 \pm 0.6 ^a	16 \pm 1 ^a	49.4 \pm 13 ^a	17 \pm 1 ^b	13.7
<i>Gao1</i>	D4.2R	21 \pm 7 ^a	29 \pm 2	316 \pm 70 ^a	30 \pm 6	15
	D4.4R	12 \pm 3 ^a	42 \pm 5 ^a	476 \pm 116 ^a	34 \pm 3	39.7
	D4.7R	15 \pm 2 ^c	38 \pm 3 ^b	398 \pm 102 ^a	39 \pm 4 ^b	26.5
	D2LR	182 \pm 42	35 \pm 2	534 \pm 67	31 \pm 2	2.9
	D2SR	24 \pm 4 ^b	36 \pm 1	159 \pm 26 ^b	36 \pm 2	6.6
<i>Gao2</i>	D3R	2 \pm 0.3 ^b	22 \pm 1 ^b	33 \pm 11 ^b	21 \pm 2	16.5
	D4.2R	197 \pm 45	53 \pm 3 ^c	408 \pm 118	54 \pm 4 ^a	2.1
	D4.4R	225 \pm 44	57 \pm 3 ^a	403 \pm 78	54 \pm 3 ^a	1.8
	D4.7R	228 \pm 38	60 \pm 4 ^a	557 \pm 114	58 \pm 4 ^a	2.4
	D2LR	227 \pm 41	64 \pm 1	727 \pm 53	55 \pm 2	3.2
<i>Gao2</i>	D2SR	49 \pm 14 ^a	64 \pm 2	298 \pm 68 ^a	60 \pm 3	6.1
	D3R	3 \pm 0.4 ^a	33 \pm 2 ^a	49 \pm 19 ^a	30 \pm 3 ^a	16.3
	D4.2R	36 \pm 16 ^a	72 \pm 6	81 \pm 26 ^a	61 \pm 5	2.3
	D4.4R	50 \pm 14 ^a	69 \pm 4	275 \pm 55 ^a	63 \pm 3	5.5
	D4.7R	128 \pm 51 ^b	63 \pm 5	122 \pm 36 ^a	57 \pm 5	0.95

ND, not determined; NE/DA, ratio between EC₅₀ values of norepinephrine and dopamine.

^a*P* < 0.001 compared with D2LR (one-way analysis of variance, followed by post hoc Newman-Keuls test).

^b*P* < 0.05 compared with D2LR (one-way analysis of variance, followed by post hoc Newman-Keuls test).

^c*P* < 0.01 compared with D2LR (one-way analysis of variance, followed by post hoc Newman-Keuls test).

analyzed. RLuc8-fused receptor constructs (Urizar et al., 2011) were transfected with Venus-fused β arrestin-2 and GRK2, whose phosphorylation precedes and assists β arrestin recruitment (Evron et al., 2012). Although right-shifted compared with the G protein-related assays, the EC₅₀ values were in agreement with previous reports (Namkung et al., 2009; Clayton et al., 2014). Notably, consistent with the radioligand-binding and G protein-related experiments, the rank order of potency was D3R > D4R > D2R for both dopamine and norepinephrine, and no variant differences were observed (Fig. 3, Table 4). The *E*_{max} values for both dopamine and norepinephrine were very similar across all dopamine receptor subtypes (Table 4).

Norepinephrine Activates G Protein, Inhibits Adenylyl Cyclase, and Promotes β Arrestin-2 Recruitment via α 2AR at Concentrations 20-Fold Lower Than D₂-Like Receptors. The α 2AR is a canonical Gi-coupled receptor for norepinephrine that is highly expressed in the prefrontal cortex (Wang et al., 1996) where D2R and D4R also are expressed (Missale et al., 1998). The EC₅₀ values of norepinephrine for the G protein activation and adenylyl cyclase inhibition assays were both at the nanomolar range and just about 20-fold lower compared with those of D3R and D4R (Fig. 4, Table 5). Similarly, the potency of norepinephrine for β arrestin-2 recruitment with α 2AR was 4-fold higher than

with D₂-like receptors, in agreement with their potency separation on G protein activation (Fig. 4, Table 5).

Discussion

From all D₂-like receptor subtypes, to our knowledge, only D4R has been suggested to be a “promiscuous” receptor that can be activated by both dopamine and norepinephrine and function as an adrenergic receptor in the brain (Lanau et al., 1997; Newman-Tancredi et al., 1997; Czermak et al., 2006; Cummings et al., 2010; Root et al., 2015). Our present study indicates that all D₂-like receptor subtypes can bind with high affinity and be activated by norepinephrine and that their potencies, particularly for D3R and D4R, are only ~20-fold lower than for α 2AR activation.

Application of BRET assays that allow measuring activation of specific G protein subtypes has provided evidence for a significant differential dependence on *Gai/o* protein subunits on the ability of both catecholamines to activate D₂-like receptors. Relative potencies obtained with the four assays used in this study were largely consistent, verifying the validity of potencies for the norepinephrine-mediated G protein activation. Furthermore, the study does not support differences in the ability of dopamine or norepinephrine to

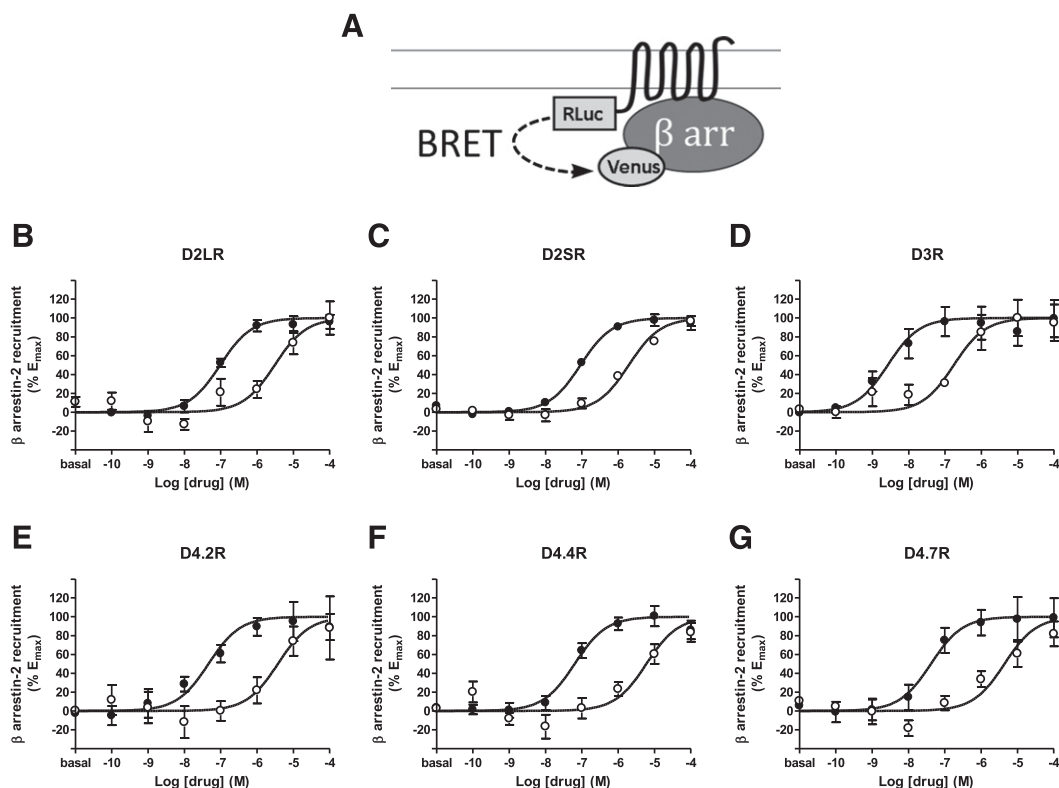


Fig. 3. (A) Scheme of the constructs used for β arrestin-2 recruitment-BRET experiments with different D₂-like receptors fused to RLuc8 and β arrestin-2 fused to mVenus. (B–G) Dose–response experiments of β arrestin-2 recruitment by dopamine (●) or norepinephrine (○) mediated by (B) D2LR, (C) D2SR, (D) D3R, (E) D4.2R, (F) D4.4R, or (G) D4.7R in HEK-293T cells transiently expressing the indicated D₂-like receptor fused to RLuc8, β arrestin-2-mVenus, and GRK2. Cells were treated with coelenterazine H followed by increasing concentrations of dopamine or norepinephrine. After 20 minutes, BRET was measured as described in *Materials and Methods*. BRET values in the absence of ligands were subtracted from the BRET values for each agonist concentration. Data were fit by nonlinear regression to a sigmoidal dose–response relationship against the agonist concentration and are shown as a percentage of the maximal effect. Data are the mean \pm S.E.M. of 5 to 10 experiments performed in triplicate (see Table 4 for EC₅₀ and E_{max} values and statistical comparisons).

activate the three main D4R polymorphic variants but does support differences in the two D2R isoform variants for their ability to couple with specific G α i/o protein subunits.

TABLE 4

β arrestin-2 recruitment induced by dopamine and norepinephrine mediated by D2LR, D2SR, D3R, D4.2R, D4.4R, and D4.7R

Results were obtained from β arrestin-2 recruitment-BRET experiments in HEK-293T cells transiently transfected with different D₂-like receptors fused to RLuc8 and β arrestin-2 fused to mVenus. Data of individual experiments were fit by nonlinear regression to a sigmoidal dose–response relationship against the agonist concentration. The EC₅₀ and E_{max} values are the mean \pm S.E.M. of 5 to 15 experiments performed in triplicate. E_{max} values are expressed as 1000 \times [BRET_{max} – BRET_{basal}].

Receptor	Dopamine		Norepinephrine		NE/DA (EC ₅₀)
	EC ₅₀ (nM)	E _{max}	EC ₅₀ (nM)	E _{max}	
D2LR	96 \pm 16	11 \pm 1	4800 \pm 1720	11 \pm 2	50
D2SR	105 \pm 21	18 \pm 2 ^a	2880 \pm 1150	17 \pm 2	27.4
D3R	3 \pm 1 ^b	15 \pm 2 ^c	32 \pm 5 ^c	13 \pm 2	10.7
D4.2R	38 \pm 13 ^c	9 \pm 1	3750 \pm 1620	10 \pm 2	100
D4.4R	66 \pm 13	8 \pm 1	2830 \pm 666	7 \pm 1	43
D4.7R	44 \pm 12 ^c	9 \pm 1	1840 \pm 889	8 \pm 2	41.8

NE/DA, ratio between EC₅₀ values of norepinephrine and dopamine for each receptor.

^aP < 0.01, compared with D2LR (one-way analysis of variance, followed by post hoc Newman-Keuls test).

^bP < 0.001, compared with D2LR (one-way analysis of variance, followed by post hoc Newman-Keuls test).

^cP < 0.05, compared with D2LR (one-way analysis of variance, followed by post hoc Newman-Keuls test).

Several studies have reported that norepinephrine can bind with high affinity and activate D4R with lower potency compared with dopamine in mammalian transfected cells. The difference in agonist potency varied significantly depending on the study, ranging from 2 to more than 50 times (Lanau et al., 1997; Newman-Tancredi et al., 1997; Czermak et al., 2006; Cummings et al., 2010). Part of this variability could certainly be related to methodologic differences in radioligand binding, G protein activation, or effector activation assays used. But our study highlights the importance of the G α i/o protein subunit involved in D4R activation. Thus, with the three main human D4R polymorphic variants—D4.2R, D4.4R, and D4.7R—dopamine was approximately 10 times more potent than norepinephrine at activating G proteins containing G α i1, G α i2, or G α i3 subunits; it was only twice as potent at activating G proteins containing G α o1 or G α o2 subunits. No statistically significant differences with the potencies of dopamine or norepinephrine could be observed among the three D4R polymorphic variants coupled to any of the Gi/o protein subtypes. This extends previous results showing no differences in the ability of dopamine to activate the three different D4R variants reconstituted with purified G proteins containing G α i1, G α i2, or G α i3 subunits (Kazmi et al., 2000), which questions the studies that have suggested D4R variant-dependent differences in the potency of dopamine or norepinephrine to activate G protein ([³⁵S]GTP γ S binding; Czermak et al., 2006) or to inhibit adenylyl cyclase (Asghari et al., 1995). As expected, we did not find any difference in the ability of dopamine or norepinephrine to

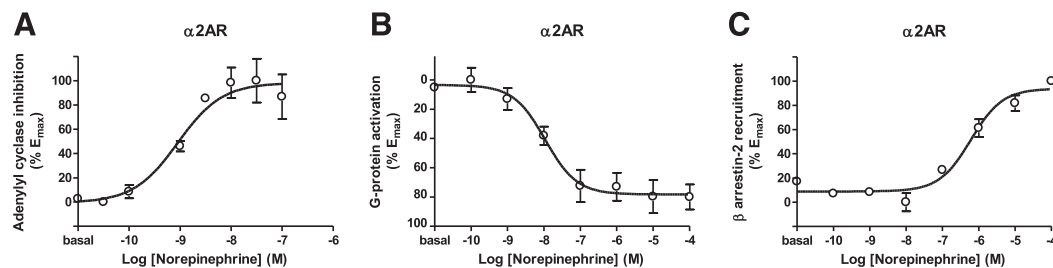


Fig. 4. (A–C) Dose–response experiments of norepinephrine on $\alpha 2AR$ function. (A) Adenylyl cyclase inhibition assay using CAMYEL biosensor. (B) G protein activation assay using $G\alpha i1$ and $\gamma 2$ subunits. (C) β Arrestin-2 recruitment assay using β arrestin-2-mVenus and GRK2. Conditions for each assay are identical to the ones described in Figures 1–3. Basal BRET values for forskolin alone (A) or in the absence of ligands (B, C) were subtracted from the BRET values for each agonist concentration. Data were fit by nonlinear regression to a sigmoidal dose–response relationship against the agonist concentration and are shown as a percentage of the maximal effect. Data are the mean \pm S.E.M. of three to four experiments performed in triplicate (see Table 5 for EC_{50} and E_{max} values and statistical comparisons).

inhibit adenylyl cyclase or to promote β arrestin recruitment between cells transfected with D4.2R, D4.4R, or D4.7R.

Previous studies reported a low potency of norepinephrine to bind and activate D2R (Lanau et al., 1997; Newman-Tancredi et al., 1997); however, after analyzing agonist binding, G protein activation, adenylyl cyclase inhibition, and β arrestin recruitment, we provide clear evidence for a similar catecholamine “promiscuity” of D2R and D3R as for D4R. The same as for D4R, Gai/o protein subunit-dependent differences were demonstrated in the potencies of dopamine and norepinephrine for D2SR, D2LR, and D3R. The most striking finding was the selective inability of dopamine or norepinephrine to activate G proteins containing the $Gai2$ subunit in D3R-transfected cells, which agrees with previous results from experiments with receptor-G protein fusion proteins and pertussis toxin-resistant mutants of G proteins containing different Gai or Gao subunits (Lane et al., 2008). Lane and colleagues reported a very efficient agonist-induced coupling of D3R to $Gao1$ and an inability of D3R to couple not only to $Gai2$ but also to $Gai1$ or $Gai3$. In our study we were also able to confirm a high potency of dopamine (and norepinephrine) to induce D3R-mediated coupling to $Gao1$ and $Gao2$ as well as to $Gai1$ and $Gai3$. Different methodologies should explain the discrepancies between both studies, but in support of our results not only dopamine but also norepinephrine were more potent at D3R than at D2LR or D2SR to induce $Gai1$ and $Gai3$ activation.

Furthermore, our results with G protein activation are congruent with our signaling experiments because HEK-293 cells have been reported to express predominantly $Gai1$, $Gai2$, and $Gai3$ and statistically nonsignificant levels of $Gao1$ and $Gao2$ (Atwood et al., 2011). Thus, both dopamine and norepinephrine were more potent at D3R than at D2LR or D2SR to

inhibit adenylyl cyclase or recruit β arrestin. Even though G proteins of the $G\alpha s$ - $G\alpha olf$ family do show contrasting brain expression pattern (Hervé 2011), to our knowledge no clear region-specific pattern of mRNA expression for Gai/o protein subtypes has been reported. Detailed characterization of expression patterns for Gai/o protein subtypes would be central to determine their stoichiometric relationship with the different D_2 -like receptors and thus the possible contribution of dopamine and norepinephrine in their activation.

Although still a matter of intense debate, as for the human D4R polymorphic variants there is no consensus about the main functional differences between the two D2R isoform variants (Gardner et al., 1996; Xu et al., 2002). Nevertheless, two research groups have independently reported a differential dependence of D2LR and D2SR on G protein subtypes for agonist-induced signaling. Specifically, D2LR has been reported to interact efficiently with the $Gai2$ subunit and poorly with the $Gai1$ and $Gai3$ subunits, while D2SR does not discriminate between these three G protein subunits (Montmayeur et al., 1993; Liu et al., 1994; Guiramand et al., 1995). It has also been suggested that the differential G protein subunit dependence is also effector dependent. Thus, G proteins containing $Gai1$, $Gai2$, or $Gai3$ subunits would selectively couple and inhibit adenylyl cyclase whereas G proteins containing Gao subunits would couple and close calcium channels (Liu et al., 1994). In our study, we did not find an absolute selective ability of D2LR to activate $Gai2$ -containing G protein, but a relative $Gai2$ preference compared with D2SR and a higher potency of dopamine and norepinephrine at D2SR compared with D2LR were observed with most Gai/ao subunits. The concomitant expression of $Gai1$, $Gai2$, and $Gai3$ subunits in HEK-293 could explain the higher potency of dopamine at D2SR to inhibit adenylyl cyclase, compared with D2LR (Montmayeur et al., 1993).

The $\alpha 2AR$ is functionally relevant to the D_2 -like receptors among the canonical norepinephrine adrenergic receptors because it is coupled to Gi/o proteins and highly expressed in the prefrontal cortex (Wise et al., 1997) where D_2 -like receptors are also expressed, especially D2R and D4R (Missale et al., 1998). The $\alpha 2AR$ is considered a high-affinity receptor for norepinephrine with a reported K_i value of as low as 15 nM in transfected cells (O'Rourke et al., 1994). This is only 3- to 5-fold higher affinity compared with the K_i values of norepinephrine for D_2 -like receptor reported herein. Our functional readouts of Gi activation, adenylyl cyclase inhibition, and β arrestin recruitment showed greater but still relatively

TABLE 5

Adenylyl cyclase inhibition, G-protein activation and β arrestin-2 recruitment induced by norepinephrine mediated by $\alpha 2AR$

Data of individual experiments were fit by nonlinear regression to a sigmoidal dose–response relationship against the agonist concentration. The EC_{50} and E_{max} values are the mean \pm S.E.M. of 3 to 4 experiments performed in triplicate. E_{max} values are expressed as $1000 \times [BRET_{max} - BRET_{basal}]$.

BRET Assay	Norepinephrine	
	EC_{50} (nM)	E_{max}
Adenylyl cyclase inhibition	1 \pm 0.2	76 \pm 10
G-protein activation ($Gi1$)	12 \pm 2	24 \pm 2
β Arrestin-2 recruitment	687 \pm 247	15 \pm 1

small separation between the potencies of norepinephrine and dopamine for α 2AR and D₂-like receptors (20-fold), verifying D₂-like receptors as potential signal transducers for norepinephrine.

With our findings on relative potencies, the previously reported so-called cross reactivity phenomenon between dopamine and norepinephrine (Guiard et al., 2008; Gonzalez et al., 2012a; Lei, 2014) can now be better explained at the receptor-effector levels and considered as likely biologically occurring events. Furthermore, dopamine and norepinephrine concentrations in prefrontal cortex are comparable (Koob et al., 1975; Blank et al., 1979; Li et al., 1998), suggesting that actions of norepinephrine at cortical D₂-like receptors are likely functionally significant.

In conclusion, norepinephrine is a potent agonist for D₂-like receptors and, like dopamine, follows a general rank order of potency of D3R > D4R \geq D2SR \geq D2L in most functional assays. If D4R functions as an adrenergic receptor in some brain regions (Root et al., 2015), the other D₂-like receptors—which in fact can be expressed with higher density in some of those areas—should also be considered as functional adrenergic receptors. Some intriguing differences in G protein activation have emerged, which depend on the G α i and G α o subunits involved. Although the framework of similarity among different effectors as well as between the two catecholamines may serve as a foundation for future studies on modulatory effects such as protein–protein interactions, the dissimilarities may be potentially pursued for further pharmacologic investigations.

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Authorship Contributions

Participated in research design: Sánchez-Soto, Ferré, Yano.

Conducted experiments: Sánchez-Soto, Bonifazi, Cai, Ellenberger, Yano.

Contributed new reagents or analytic tools: Yano.

Performed data analysis: Sánchez-Soto, Bonifazi, Cai, Ellenberger, Newman, Ferré, Yano.

Wrote or contributed to the writing of the manuscript: Sánchez-Soto, Bonifazi, Newman, Ferré, Yano.

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