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Evidence against dopamine D1/D2 receptor heteromers

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Abstract

Hetero-oligomers of G-protein-coupled receptors have become the subject of intense investigation because their purported potential to manifest signaling and pharmacological properties that differ from the component receptors makes them highly attractive for the development of more selective

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pharmacological treatments. In particular, dopamine D1 and D2 receptors have been proposed to form hetero-oligomers that couple to $G_{\alpha q}$ proteins, and SKF83959 has been proposed to act as a biased agonist that selectively engages these receptor complexes to activate $G_{\alpha q}$ and thus phospholipase C. D1/D2 heteromers have been proposed as relevant to the pathophysiology and treatment of depression and schizophrenia. We used *in vitro* bioluminescence resonance energy transfer (BRET), *ex vivo* analyses of receptor localization and proximity in brain slices, and behavioral assays in mice to characterize signaling from these putative dimers/oligomers. We were unable to detect $G_{\alpha q}$ or $G_{\alpha 11}$ protein coupling to homomers or heteromers of D1 or D2 receptors using a variety of biosensors. SKF83959-induced locomotor and grooming behaviors were eliminated in D_1 receptor knockout mice, verifying a key role for D1-like receptor activation. In contrast, SKF83959-induced motor responses were intact in D2 receptor and $G_{\alpha q}$ knockout mice, as well as in knock-in mice expressing a mutant Ala^{286}-CaMKIIα, that cannot autophosphorylate to become active. Moreover, we found that in the shell of the nucleus accumbens, even in neurons in which D1 and D2 receptor promoters are both active, the receptor proteins are segregated and do not form complexes. These data are not compatible with SKF83959 signaling through $G_{\alpha q}$ or through a D1–D2 heteromer and challenge the existence of such a signaling complex in the adult animals that we used for our studies.

Keywords

dopamine; $G_{\alpha q}$; biased agonism; hetero-oligomer; D1; D2; D5; BRET; proximity ligation assay; striatum

INTRODUCTION

Increasing evidence suggests that G protein-coupled receptors (GPCR) can form dimers and/or oligomers with properties distinct from those of the component receptors. These altered properties include allosteric modulation between protomers, altered affinity for ligands, differential coupling to downstream signaling pathways, and cross-regulation of receptor trafficking[1, 2]. These findings are of great interest since they suggest new routes to the development of selective ligands that could selectively target specific GPCR complexes with reduced off-target effects. However, most of the evidence supporting the formation of GPCR dimers and oligomers has come from heterologous systems, and both the existence of such signaling complexes in the native context and their biological significance remain controversial[3]. In particular, it is extremely difficult to dissociate downstream crosstalk from the actual physical interaction of two receptors in a signaling complex[4, 5].

Heteromers putatively formed by dopamine (DA) D1 and D2 receptors have been the subject of intense research because of their unusual signaling properties and potential implication in various pathologies. Although D1 and D2 receptors are largely segregated in neurons of the striatal direct and indirect pathways, respectively[6, 7], they have been reported to be colocalized in a subset of ventral striatal neurons, where they have been proposed to form a third striatal output pathway[8–13].

Classically, these receptors are thought to mediate cyclic-AMP-dependent signaling through the activation of $G_{\alpha s/olf}$ or $G_{\alpha i/o/z}$ by D1 and D2 receptors, respectively[14]. It has also been

reported that specific D1-like receptor ligands can activate alternative signal transduction systems resulting in phosphatidylinositol (PI) hydrolysis and accumulation of inositol triphosphate in the amygdala, hippocampus, striatum and frontal cortex[15–18]. Notably, SKF83959, a D1-like receptor partial agonist has been reported to activate D1-like receptors linked to stimulation of PI hydrolysis[19–21] but to only have minimal or even antagonistic effects on the activation of adenylyl cyclase[19, 20, 22] (but see[23, 24]). This compound has been inferred to selectively mediate phospholipase C (PLC) activation through $G_{\alpha q}$ recruitment to D1/D2 receptor heteromers[25]. The resulting intracellular calcium mobilization has been proposed to mediate D1/D2 heteromer-specific activation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKIIα)[10, 25, 26].

Although the biological significance of signaling mediated by the proposed D1/D2 receptor heteromer remains largely unknown, the complex has fascinated the field because of its potential involvement in pathological conditions including schizophrenia[9] and depression[27], as well as its unique pharmacological properties that could allow for the development of highly selective compounds. D1/D2 heteromers have been implicated in a variety of physiological processes such as BDNF expression[28], neuronal growth[28], modulation of fibroblast growth factor (FGF)-2 in astrocytes[29], inhibition of high-voltage Ca^{2+} currents in striatal cultures[30], facilitation of long-term depression in the hippocampus[31] and spontaneous glutamate release in the cortex[32].

Despite these exciting findings, there are inconsistencies in the literature that are difficult to reconcile with these proposed functions of D1/D2 heteromers and necessitate further investigation. In particular, using global knockout mice, D1-like receptor-dependent stimulation of PI hydrolysis has been shown to be independent of D1 receptors but rather to depend on D5 receptors[33, 34], and a requirement for D1/D2 heteromers in SKF83959-induced calcium mobilization has recently been challenged[23, 35]. Here, we assessed the ability of DA receptors to recruit $G_{\alpha q}$ signaling both *in vitro* and *in vivo*. First, using bioluminescence resonance energy transfer (BRET) and complemented donor acceptor-RET (CODA-RET), we systematically investigated DA receptor activation of specific G-proteins *in vitro*. Next, we used behavioral pharmacologic approaches in mutant mouse models to assess the roles of functional DA receptors, $G_{\alpha q}$, and CaMKII in the behavioral responses induced by SKF83959. Finally, we analyzed the extent of colocalization of D1 and D2 receptors *ex vivo* by immunohistochemistry coupled with the identification of cells co-expressing both receptors in Drd1a-tdTomato/Drd2-eGFP double BAC transgenic mice. We also measured *ex vivo* the level of interaction of endogenous D1 and D2 receptors using a proximity-ligation assay (PLA). Our findings challenge the existence of an atypical $G_{\alpha q}$ signaling pathway activated by D1/D2 heteromers *in vitro* and *in vivo*, as well as the existence of such complexes *in vivo* at endogenous receptor expression levels.

MATERIALS AND METHODS

HEK Cell Culture

The cDNAs for the human DA D1 receptor, D5 receptor, muscarinic M1 receptor, 5HT2A receptor, and $G_{\alpha s}$ short were obtained from www.cdna.org. The D1, D5, and M1 receptors were tagged at their amino termini with a signal peptide[36] followed by a Myc epitope tag

using standard molecular biology procedures. The cDNAs encoding full length Renilla Luciferase 8 (RLuc8) or fragments for the Luciferase 1 (L1: a.a. 1–229) or Luciferase 2 (L2: a.a. 230–311) were fused in frame to the C-terminus of D1R, D5R, M1R, or 5HT2AR in the pcDNA3.1 vector. The sensors for the human D2 receptor short isoform (D2SR) have been previously reported[37] and constructs for the D2 receptor long isoform (D2LR) were made accordingly. These D2 receptor constructs were N-terminally fused to a signal peptide followed by a flag epitope.

The following human G-protein constructs were used: $G_{\alpha q}$-mVenus with mVenus inserted at position 97, $G_{\alpha i}$-RLuc8 with RLuc8 inserted at position 91, $G_{\alpha s}$ short-RLuc8 with RLuc8 inserted at position 67, $G_{\alpha q}$-RLuc8 with RLuc8 inserted at position 97, $G_{\alpha 11}$-Rluc8 with RLuc8 inserted at position 92, untagged $G_{\beta 1}$, untagged $G_{\gamma 2}$ and $G_{\gamma 2}$ fused to full-length mVenus or GFP10 at its N-terminus. All the constructs were confirmed by sequencing analysis. A constant amount of plasmid cDNA (15 μg) was transfected into HEK-293T cells using polyethylenimine (PEI) (Polysciences Inc, Warrington, PA) in a 1 to 3 ratio in 10 cm dishes. Cells were maintained in culture with DMEM supplemented with 10% FBS. The transfected amount and ratio among the receptor-L1, receptor-L2, G_{α}, $G_{\beta 1}$, $G_{\gamma 2}$ for complemented donor acceptor RET (CODA-RET) or BRET between Gα and Gγ subunits was optimized for maximal dynamic range for drug-induced BRET. Experiments were performed approximately 48 hours post-transfection.

BRET

BRET1 uses a yellow fluorescent protein variant (mVenus) as an acceptor for energy transfer from luciferase and was measured as described[37]. BRET2 uses a green fluorescent protein variant (GFP10) as an acceptor for energy transfer from luciferase and was performed as described[38]. Briefly, cells were harvested, washed and resuspended in PBS. Approximately 200,000 cells/well were distributed in 96-well plates and 5 μM coelenterazine H (substrate for luciferase in BRET1) or 5 μM deep blue c (substrate for RLuciferase8 in BRET2) was added to each well. Luciferase substrates were added to each well 5 minutes prior to detection. Typically antagonist was added 15 min before the addition of agonist. The fluorescence (excitation at 500 nm and emission at 540 nm for 1 sec recording in BRET1 or excitation at 410 nm and emission at 510 nm for 1s recording in BRET2) and luminescence (no filters, 1 sec recording) were detected 2.5 min after a ligand was added (Polarstar, Pherastar, or Pherastar FS BMG). In parallel, the BRET signal from the same batch of cells was determined by quantifying and calculating the ratio of the light emitted by mVenus (510–540 nm) over that emitted by RLuc8 or RLuc (485 nm) for BRET1 and the ratio of the light emitted by GFP10 (515 nm) over that emitted by RLuc8 (370–450 nm) for BRET2. The net BRET values were obtained by subtracting the background determined in cells expressing RLuc8 or RLuc alone. Results are expressed as the BRET change produced by the corresponding drug. As shown in the cartoons, bimolecular complementation of donor was incorporated into the BRET assay. RET took place between complemented luciferase complex and G_{α}-mVenus.

Measurement of Calcium Flux in Stably Expressed Cell Lines

Stable cell lines expressing D1, D2S, or D1 + D2S were generated as previously described[4]. Surface expression of HA-tagged D1 and FLAG-tagged D2S receptors was confirmed by fluorescence-activated cell sorting (data not shown). Calcium flux was measured using the Flipr Calcium 5 Assay kit (Molecular Devices, Sunnyvale, CA) according to the manufacturer's protocol. Briefly, cells were resuspended in HBSS buffer containing 20 mM HEPES and 2.5 mM probenecid and distributed in 40 μl volumes in 96 well plates (500,000 cells/well). Fifty μl Flipr5 dye (Molecular devices) was added to each well and plates were incubated in 37°C 5% CO2 for 1 hour. Plates containing 10x concentrated ligand were prepared in HBSS with 20 mM HEPES. During the calcium reading, performed on a Flexstation 3 (Molecular Devices), 10 μl ligand was injected to the well at the indicated times. Intracellular calcium levels were measured every 2 sec over the course of ~110 sec. Data was analyzed by Softmax Pro 5.4 (Molecular Devices) and Prism 5.0 (Graphpad, La Jolla, CA).

Animals

C57Bl/6J mice (Jackson, 9–12 weeks old) were utilized for dose-response experiments, which examined the effects of dopaminergic antagonists on SKF83959-mediated behaviors. For behavioral experiments, D1[39], D2[40] and D5 receptor[41] knockouts, $G_{\alpha q}$ knockouts and CaMKIIα-Thr^{286}Ala knockin mice were bred at Vanderbilt University using strategies previously described for generation of mixed litters and assignment of genotypes[42]. All lines were fully backcrossed (>10 generations) to a C57Bl/6J background.

Twelve-week old D2[40] and D1[39] receptor knockout (KO) and their C57Bl/6J wildtype (WT) littermates were used for immunohistochemical experiments. Twelve to sixteen week old C57Bl/6J mice (Jackson) were used for viral injections (see below).

Male mice were housed under standard housing conditions on a 12 h light/dark cycle with conditions previously described[42]. Mice were extensively handled prior to testing and were habituated to the testing rooms for ~30 min prior to beginning of every experiment. All procedures were approved by the Institutional Animal Care and Use Committees at Vanderbilt University or Columbia University.

For visualization of D1- and D2- expressing neurons, Drd1a-tdTomato (Tg(Drd1a-tdTomato)5Calak)[43] and GENSAT Drd2-eGFP (Tg(Drd2-EGFP)S118Gsat/Mmnc) mice[44] were used. The lines were intercrossed to report the gene expression patterns of the D1 and D2 receptors in the same animal. Male and female breeders, each hemizygous for one of the aforementioned transgenes, were bred to attain mice that were singly hemizygous for both Drd1a-tdTomato and Drd2-eGFP. Genotypes were confirmed via PCR[43] (see MMRRC web site for detailed PCR conditions). Similar results were obtained when a brighter Drd1a-tdTomato line, the Tg(Drd1a-tdTomato)6Calak)[45], was crossed with the Drd2-eGFP mice. This latter double-transgenic line was used for counting of tdTomato/eGFP-positive cells.

Locomotor Behavior and Grooming

Motor responses were measured using commercial open field activity chambers (Med Associates, 29 × 29 × 20.5 cm) that were contained within light- and air-controlled environmental chambers (Med Associates, St. Albans, VT; 64 × 45 × 42 cm). Location and movement were detected by the interruption of infrared beams. A three-day protocol was employed where the mice received injections of 0.9% saline for two days and SKF83959 on the third day as previously described[42]. Following the SKF83959 injection, the mice were placed in the activity chambers for 60 min. Data presented are from this third session, following repeated habituation to the chambers. For bar graph displays "Pre-injection" refers to the average ambulatory distance during 5 min epochs from min 10–30 (that is, just prior to injection) and "Post-injection" to the same measure from min 40–90 (10–60 min after the injection).

During the open field testing, mice were additionally monitored by an overhead camera for analyses of grooming. Grooming was analyzed from the video recordings by assessing the number of grooming events for a 5 min period during the baseline session and 5 min from the post-injection period. The rater was blinded to genotype.

Surgeries/viral injections

Viral injections were performed as described previously[46, 47]. Adeno-associated viruses 1/2 expressing the D2L receptor fused to mVenus[46, 47] and lentiviruses expressing an untagged rat D1 receptor under the synapsin promoter were used[48]. The nucleus accumbens was targeted with a single injection site bilaterally (2 sites total, 0.5 μl for AAV1/2, 2 μl for lentiviruses injected in each site): A–P, 1.7 mm; M–L, ±1.1 mm relative to bregma; and both 3.85 mm ventral to brain surface. Animals were sacrificed 1 month after surgery.

Drugs

For the cell culture assays, the following pharmacologic reagents were used: 5-HT (Sigma, St. Louis, MO), acetylcholine (Sigma, St. Louis, MO), DA (Sigma, St. Louis, MO), the 5-HT2A/C receptor antagonist ketanserin (Sigma, St. Louis, MO), the M1 muscarinic receptor antagonist pirenzepine (Sigma, St. Louis, MO), the D2-like receptor agonist quinpirole (Tocris Biosciences, Minneapolis, MN) and the D1-like receptor agonist SKF83959 (Tocris Biosciences, Minneapolis, MN).

For the behavioral studies, SKF83959 (Tocris Biosciences, Minneapolis, MN) was dissolved in 0.9% saline solution at 0.2 mg/cc (1 mg/kg) and injected intraperitoneally (i.p.). For the dose response experiments, additional doses of SKF83959 (0.05 and 0.25 mg/kg) were also used. The D2-like receptor antagonist raclopride (Sigma, St. Louis, MO) was used at 0.5 mg/kg and the D1-like receptor antagonist SCH23390 (Sigma, St. Louis, MO) was used at 0.01 and 0.25 mg/kg.

Immunohistochemistry

Brain tissue preparation, immunohistochemistry, confocal microscopy and image acquisition were performed as described previously[46, 47]. The following primary antibodies were used: rat anti-D1 receptor antibody (1:200–1:500, Sigma Aldrich, St. Louis, MO; cat# D2944),

rabbit anti-D2 receptor antibody (1:100–1:300, see[46, 47]), anti-GFP antibody (1:500, Abcam, Cambridge, MA; cat# ab13970) and rabbit anti-dsRED antibody (1:300, Clontech, Mountain View, CA; cat# 632496). For double labeling of D1 and D2 in double BAC transgenic mice, the slices were first incubated with rabbit anti-D2 receptor, rat anti-D1 receptor and chicken anti-GFP antibodies followed by appropriate secondary antibodies. The tomato signal was then enhanced using rabbit anti-DsRed antibody that was directly labeled with Alexa 568 using APEX Alexa Fluor 568 Antibody Labeling Kit (Invitrogen; A10494). The following secondary goat antibodies from Invitrogen were used: anti-rabbit Alexa 568 or 405, anti-rat Alexa 647, anti-chicken 488, all at a concentration of 1/1000.

Proximity-Ligation Assay (PLA)

PLA was performed using the Duolink *in situ* kit (Olink Bioscience) according to the manufacturer's instructions with the following modifications: Incubation with PLA probes was for 2 hours at 37°C; ligation step was performed for 45 min at 37°C; amplification step was extended to 2 hours at 37°C with a concentration of polymerase of 1/60. Anti-rat PLA probes were generated according to the manufacturer's instructions using the Duolink Probemaker (Olink Bioscience) and goat-anti rat IgGs (Santa Cruz).

Image Processing and Quantification of PLA signal

To process and quantify the PLA signal we used a method described in detail in Biezonski et al. (under review). All images were loaded into MATLAB (The MathWorks, Inc.) and converted from 12-bit into double precision intensity values ranging from 0 to 1. The resolution of all images was 210 nm/pixel. To develop a database for representative PLA signal for our images, individual signal was first selected from three positive control images (D1D2 overexpression). Approximately 70 such areas that ranged in size from 5 pixels to 45 pixels were chosen from each of three images. Similarly, a number of background regions were chosen from negative control images (D1 and D2 KO). To achieve the greatest separation between the DuoLink signal and the background, we standard deviation filtered all the images and analyzed the standard deviation values within our pre-selected regions. The DuoLink signal and background were conservatively separable at 0.08 units of standard deviation. This threshold captured 95% of all identified signal pixels and only 40% of identified background pixels. Of these background pixels derived from the negative control image, 85% belonged to connected objects that were below 5 pixels in size. Thus, we applied a size threshold of 5 pixels for all DuoLink signal and this way excluded approximately 50% of the background pixels. In parallel, to treat the confounding areas of high fluorescence intensity within nuclei, we entropy filtered all of the intensity images with a filter size of our minimum particle size (5 pixels). Within the entropy image, nuclei contained uniformly high values while smaller signals receded into the background. This allowed us to exclude any fluorescence originating within nuclei from the ultimate analysis of the DuoLink signal. All standard deviation filtered images were then thresholded at 0.08 units of standard deviation and the surviving pixels formed masks. Any mask was excluded if its size was below 5 pixels. The masks created from these thresholded images represented the final DuoLink signal.

Data Analysis and Statistics

Data and statistical analysis were performed with Prism 5.01 (GraphPad Software, San Diego, CA) or with SPSS version 22 (IBM, Armonk, NY). For BRET studies, dose response curves of agonist response and non-responsive counterpart (i.e. antagonist treatment or non-responsive G protein coupling) were examined with an F-test analysis. Behavioral data were subjected to one- or-two way analysis of variance (ANOVA) with genotype as a between-group factor. Post-hoc Tukey's multiple comparison tests or Bonferroni comparisons were used to compare groups to each other. PLA data were analyzed using unpaired t-tests. Graphs are marked with asterisks (*$p<0.05$, **$p<0.01$, ***$p<0.001$) to denote statistical significance.

RESULTS

D1 and D2 receptors expressed individually activate their cognate G proteins but do not activate $G_{\alpha q}$

Activation of G-proteins by specific DA receptor populations was systematically assessed using the BRET biosensor technique (Figure 1a). This approach allows measurement of G-protein activation based on a conformational change between the α and γ subunits (Figure 1a). The conformational changes assessed by the sensors have been previously demonstrated to accurately reflect activation of the G proteins[38, 49].

DA receptor activation of $G_{\alpha q}$ was assessed with either DA or the agonist SKF83959. We failed to detect any drug-induced BRET changes in the $G_{\alpha q}$ biosensor in response to DA or SKF83959 stimulation of D1 or D2long (D2L) (Figure 1b) or from D5 or D2short (D2S) receptors (Supplementary Figure 1b). To demonstrate the functionality of the $G_{\alpha q}$ biosensor[50], the M1 muscarinic receptor, a $G_{\alpha q}$-coupled receptor, was expressed and stimulated by acetylcholine (Figure 1b), which resulted in a robust concentration-dependent activation of BRET. As expected, acetylcholine-induced activation was inhibited by the selective antagonist pirenzepine (Figure 1b).

In contrast, robust cognate G-protein activation (i.e. D1-$G_{\alpha s}$, D5-$G_{\alpha s}$, D2S-$G_{\alpha i1}$ and D2L-$G_{\alpha i1}$) was detected by a change in BRET between the α and γ subunits after stimulation with DA (Figure 1c and d for D1-$G_{\alpha s}$ and D2L-$G_{\alpha i1}$, Supplementary Figure 1c and d for D5-$G_{\alpha s}$ and D2S-$G_{\alpha ii1}$). These signals were inhibited, as expected, by the appropriate antagonists (Figure 1c and d, Supplementary Figure 1c and d).

D1 and D2 receptors expressed together fail to activate $G_{\alpha q}$

Both D1/D2 receptor[25] and D5/D2 receptor[51] heteromers have been reported to couple to $G_{\alpha q}$ activation, and D1 and D2 receptors when coexpressed led to mobilization of calcium in response to DA (but not SKF83959)[35]. Therefore, $G_{\alpha q}$ activation was tested after co-expression of the unfused DA receptors with the $G_{\alpha q}$ biosensor proteins (Figure 1e and Supplementary Figure 1e). Neither stimulation by DA nor SKF83959 led to $G_{\alpha q}$ activation in cells co-expressing non-fused receptors – D1+D2L receptors (Figure 1f), D5+D2L (Supplementary Figure 1f), D1+D2S receptors (Supplementary Table 1), or D5+D2S receptors (Supplementary Figure 1g). As a positive control, the M1 receptor was co-

expressed with DA receptors. Drug-induced BRET mediated by the M1 receptor was not altered in the presence of D2L or D2S, indicating that the co-expression did not impair the function of the $G_{\alpha q}$ biosensor (Figure 1f and Supplementary Figure 1f and g, blue plot).

When two receptors are co-expressed, it is not possible to differentiate signaling between individual protomers, homomers, or heteromers. Therefore we used the CODA-RET configuration[5] (Figure 2a), to measure $G_{\alpha q}$-coupling specifically from the defined heterodimer. In this method, split luciferase is fused to the C terminus of two receptors, and luminescence only results from complementation of the luciferase due to association of the receptors bearing the halves. Thus, BRET between receptor and an acceptor-tagged G_{α} is indicative of signaling of the defined dimer through a defined G protein[5]. Homodimer complementation of the prototypical $G_{\alpha q}$-coupled M1 receptor (Figure 2b) or 5HT2A receptor (Figure 2c) showed efficient agonist-stimulated $G_{\alpha q}$-coupling and antagonist blockade. This pair of positive controls showed that complemented luciferase does not interfere with $G_{\alpha q}$-coupling, at least in the case of these homodimers. In contrast, DA stimulation alone or SKF83959 and quinpirole co-stimulation failed to induce $G_{\alpha q}$-coupling of complemented D1-D2S receptors (Figure 2d), D2S-D5 receptors (Figure 2e), D1-D2L receptors (Figure 2f), or D2L-D5 receptors (Figure 2g). Despite its better sensitivity, the BRET2 configuration also failed to detect drug-induced recruitment of $G_{\alpha q}$ to either individual (Supplementary Table 1) or complemented DA receptors in any combination (Supplementary Table 1). We showed previously using CODA-RET that D1 and D2 receptors can interact in HEK cells and that signaling of the D1/D2 heteromer to $G_{\alpha i}$ is differentially altered for the agonist NPA relative to DA[5]. Thus, although these receptors can interact and modulate one another in HEK cells, our new data demonstrate that in these cells D1/D2 receptor heteromers are unable to recruit $G_{\alpha q}$ in response to DA, SKF83959, or combined quinpirole and SKF83959.

D1 and D2 receptors expressed individually or together also fail to activate $G_{\alpha 11}$

$G_{\alpha 11}$ shares significant structural and functional homology with $G_{\alpha q}$; they both couple to PLC and are expressed in the striatum. Therefore, it is possible that effects ascribed to $G_{\alpha q}$ might instead result from $G_{\alpha 11}$. We demonstrated the lack of recruitment of $G_{\alpha 11}$, in response to activation of D1, D2S, D2L, or D5 receptors (Supplementary Figure 2c and d). $G_{\alpha 11}$ activation was also tested after co-expression of the unfused DA receptors with the $G_{\alpha 11}$ biosensor proteins, and as was the case with $G_{\alpha q}$, neither stimulation by DA nor SKF83959 led to $G_{\alpha 11}$ activation in cells co-expressing non-fused receptors: D1+D2L receptors (Supplementary Figure 2f), D5+D2L (Supplementary Figure 2g), D1+D2S receptors Supplementary Figure 2f), or D5+D2S receptors (Supplementary Figure 2g). Once again, positive control experiments using M1 receptor activation verified the integrity of our $G_{\alpha 11}$ activation assay (Supplementary Figure 2b).

D1 and D2 receptors fail to induce intracellular calcium responses

At the functional level, D1/D2 heteromer-dependent activation of $G_{\alpha q/11}$ has been proposed to lead to the recruitment of calcium signaling[28, 52]. We therefore tested whether, despite the absence of $G_{\alpha q}$ or $G_{\alpha 11}$ recruitment, co-expression of D1 and D2 receptors leads to calcium mobilization (Figure 3). Neither D1 (Figure 3a) nor D2S activation (Figure 3b) in the

respective stably transfected cells caused calcium mobilization. We next created dual D1/D2S stable cells but still did not observe achange in intracellular calcium in response to D1 and D2 co-stimulation with either DA or SKF83959 (Figure 3c). In contrast, acetylcholine potently stimulated calcium via endogenously expressed muscarinic M3 receptors (Figure 3a, b, c).

The behavioral effects of SKF83959 *in vivo* depend on activation of D1 receptors but not of D2, $G_{\alpha q}$ or CaMKII phosphorylation on Thr^{286}

In order to extend our *in vitro* findings to the *in vivo* setting, we evaluated SKF83959-induced behavioral effects in mutant mice disrupted for the putative signaling mediators, D1, D5, D2, $G_{\alpha q}$ or CAMKII. In WT mice, a peripheral injection of SKF83959 (0.05–1 mg/kg) produced a dose-dependent increase in locomotor activity (Supplementary Figure 3a and b; $F(3,20)=39.9$, $p<0.001$) and a specific motor stereotypy involving orofacial grooming (Supplementary Figure 3c; $F(3,20)=39.9$, $p<0.05$), as previously described[53, 54]. The grooming response was already maximal at 0.05 mg/kg (Supplementary Figure 3c; $F(3,23)=12.8$, $p<0.001$).

That pharmacological blockade of D1 or D2 receptors blunted the SKF83959-induced behaviors (data not shown) does not establish that the drug necessarily acts directly on these receptors. For example, D2 antagonism is well known to reduce locomotor activity, and it may prevent SKF83959-induced effects indirectly. In order to assess more directly which DA receptors are necessary for mediating SKF83959-induced signaling and behavior, we investigated the SKF83959-induced grooming and locomotor activity in a panel of DA receptor knockout mice. SKF83959 elicited a significant orofacial grooming response (Figure 4a; $F(1,69)=23.6$, $p<0.001$) in WT mice but not in D1 receptor knockout mice (*post-hoc* Bonferroni multiple comparison test; $p<0.001$). We also assessed SKF83959-induced locomotion (Figure 4b; $F(1,34)=135.0$, $p<0.001$). D1 receptor knockout mice were initially hyperactive compared to their WT littermates during the pre-injection baseline session (Figure 4b and Supplementary Figure 4). During the post-injection period, WT mice increased their locomotor activity in response to SKF83959 (Figure 4b, *post-hoc* Bonferroni multiple comparison test, $p<0.001$). The D1 null mice, in contrast, did not respond to SKF83959 but continued to habituate to the chambers (Figure 4b and Supplementary Figure 4), resulting in a significant decrease in locomotor activity, as compared to pre-injection baseline (*post-hoc* Bonferroni multiple comparison test, $p<0.001$).

We next examined the effects of SKF83959 in mice lacking D2 receptors and observed significant increases in grooming in both WT and D2 null mice (Figure 4c; $F(1,33)=8.32$, $p<0.001$; *post-hoc* Bonferroni multiple comparison tests, $p<0.01$ for each). D2 receptor null mice expressed sharply reduced locomotor activity during the pre-injection period (Figure 4d, *post-hoc* Bonferroni multiple comparison test, $p<0.01$), but both the WT controls and D2 receptor −/− mice responded significantly to SKF83959 (Figure 4d; $F(1,33)=72.3$, $p<0.001$; *post-hoc* Bonferroni multiple comparison tests, $p<0.001$). Given the reduced locomotion in the D2 receptor null mice, these animals expressed an even more robust response to SKF83959 when considering the percent change from pre-injection baseline (data not shown).

We next tested a potential role for D5 receptors in the effects of SKF83959. SKF83959 significantly increased grooming ($F(3,40)=12.3$, $p<0.001$) and locomotor activity ($F(1,40)=108.6$, $p<0.001$) in D5 receptor knockout mice, although the locomotor responses were slightly reduced compared to WT (Supplementary Figure 5, *post-hoc* Bonferroni multiple comparison test, $p<0.05$).

In addition to evaluating the contribution of the DA receptors to SKF83959-mediated actions, we also assessed the contribution of the G-protein $G_{\alpha q}$ (Figure 4e, f). Similar to the D2 receptor knockouts, $G_{\alpha q}$ knockout mice were hypoactive at pre-injection baseline, but still responded robustly to SKF83959. SKF83959 significantly increased grooming in WT and $G_{\alpha q}$ knockout mice (Figure 3e, $F(1,28)=5.90$, $p<0.01$; *post-hoc* Bonferroni multiple comparison tests, $p<0.05$). Similar results were observed for locomotor activity (Figure 4f, $F(1,28)=4.99$, $p<0.01$; *post-hoc* Bonferroni multiple comparison tests, $p<0.001$).

Next we assessed the role of CaMKIIα in mediating the behavioral responses to SKF83959, as phosphorylation of CaMKIIα on Thr286 has been suggested to be a critical downstream component in the signaling of SKF83959[25, 26, 55]. To address this question we evaluated CaMKIIα – Thr^{286}Ala knockin mice, in which CaMKII is incapable of phosphorylation at the Thr^{286} residue[56, 57]. Both SKF83959-induced grooming (Figure 4g, $F(3,15)=4.99$, $p<0.01$; post-hoc Bonferroni multiple comparison tests, $p<0.05$) and locomotor activity (Figure 4h, $F(3,15)=12.2$, $p<0.01$; post-hoc Bonferroni multiple comparison tests, $p<0.001$) were fully intact in CaMKIIα – Thr^{286}Ala knockin mice.

We additionally normalized the SKF83959-induced grooming data to the pre-injection baseline observed for each mutant genotype to provide more direct comparisons within each line. As can be seen in Supplementary Figure 6, D1 receptor knockout mice expressed significantly reduced SKF83959-induced grooming as compared to controls ($p=.0126$ by unpaired *t*-test). However, no other mutant mice (D2 receptor null, $G_{\alpha q}$ null, CaMKIIα – Thr^{286}Ala knockin, D5 receptor null) differed from WT littermate controls.

D1 and D2 receptors do not form complexes in the striatum

Even if the behavioral effects of SKF83959 are not mediated by D1/D2 receptor dimers, it is conceivable that such a complex nonetheless mediates differential signaling and represents a novel target for therapeutics. In fact, despite our inability to detect the activation of $G_{\alpha q}$ by D1/D2 receptors in HEK293 cells, previously using CODA-RET we did obtain support for functional selectivity in the $G_{\alpha i}$ pathway of a defined D1/D2 receptor heteromer[5]. This piqued our interest in this potential heteromer as a drug target and motivated us to pursue studies in ventral striatal brain slices where the receptors have been reported to be co-expressed. Coexpression of D1 and D2 receptors in the striatum and nucleus accumbens has been a contentious topic[6]. *In situ* hybridization and electron microscopy studies have generally supported the segregation of these receptors in distinct subpopulations of neurons[7, 58, 59]. However, modest colocalization as well as fluorescence resonance energy transfer (FRET) between D1 and D2 receptors have been observed in the shell of the nucleus accumbens using antibodies[9, 10]. Indirect measures using BAC transgenic GFP mice support co-expression of D1 and D2 receptor genes in a sub-population of neurons in the shell of the nucleus accumbens[13, 60]. In order to clarify whether D1 and D2 receptors colocalize in the

shell of the nucleus accumbens, we took advantage of an anti-D2 receptor antibody that we previously generated and that shows high selectivity as confirmed by the lack of staining in D2 receptor-KO mice[46, 47] (Figure 5a). We detected very limited colocalization of D1 and D2 receptors in the shell of the nucleus accumbens in WT mice (Figure 5b) or rats (Supplementary Figure 7), or in the ventral striatum of rhesus monkeys (Supplementary Figure 7). Because D1 and D2 receptor subcellular localization is mostly in the neuropil[11, 46, 47, 59, 61], it is virtually impossible to visualize cell bodies that express – or not – specific dopamine receptors. In order to better identify cells that co-express both receptors we crossed two transgenic reporter lines, Drd1a-tdTomato[45] and Drd2-eGFP[44]. As previously reported based on comparing expression in the individual reporter lines[60], in the crossed lines we observed co-expression of both genes in a small fraction of cells restricted to the shell of the nucleus accumbens, but in only a handful of neurons within the NAc core or the dorsal striatum (Figure 5c).

We performed cell counts of D1-tomato+ and D2-eGFP+ cells at multiple levels throughout the rostral to caudal extent of the NAc and dorsal striatum (Supplementary Figure 8). The vast majority of cells in all regions were positive for only D1-tomato or D2-eGFP, but colocalized reporter proteins were observed in 5–7% of cells within the NAc shell (Supplementary Figure 8a). Within the NAc core, only 2–3% of reporter-expressing cells expressed both D1-tomato and D2-eGFP (Supplementary Figure 8b). The vast majority of the dorsal striatum (Supplementary Figure 8c) resembled the NAc core with only ~2% colocalization; however in the caudal tail of the striatum, colocalization increased to ~7%. We next analyzed D1 and D2 receptor expression with high resolution, selectively in those neurons that co-expressed Tdtomato and eGFP in the shell of the nucleus accumbens. Staining of D1 and D2 receptor signals in those neurons revealed complete segregation of the receptors even on dendritic segments that co-express both reporters (Figure 5d). These data suggest that even in the few neurons in which both D1 and D2 receptor promoters are active, there is little if any colocalization of the receptor proteins.

We confirmed the lack of interaction of D1 and D2 receptors using a proximity-ligation assay (PLA) that allows for the detection of receptor complexes *ex vivo*[47]. PLA signals for this heteromer were virtually absent in the nucleus accumbens of WT, D1 receptor KO and D2 receptor KO mice (Figure 6). Overexpression of either D1 or D2 receptors by viral gene transfer led to a small increase in PLA signal, whereas overexpression of both receptors resulted in a very large increase in PLA signal (Figure 6). These data suggest that even in the small fraction of neurons that co-express D1 and D2 receptors, the receptors are segregated and do not physically interact. In contrast, when both receptors are dramatically overexpressed - either in HEK cells or *in vivo* - the receptors have the ability to interact.

DISCUSSION

We were unable to detect $G_{\alpha q/11}$-coupling to monomers or heteromers of DA receptors using multiple sensitive biosensors expressed in HEK cells. SKF83959-induced locomotor and grooming behaviors were eliminated or reduced in D1 receptor knockout mice but were intact in D2 receptor and $G_{\alpha q}$ knockouts as well as in non-autophosphorylatable Ala^{286}-CaMKIIα knockin mice. These data are thus incongruent with a dependence of SKF83959-

induced behavior on D1/D2 heteromers or $G_{\alpha q/11}$ signaling. Moreover, we found that D1 and D2 receptors are segregated at the subcellular level even in medium spiny neurons in the shell of the nucleus accumbens that co-express both receptors, suggesting that these receptors do not form heteromers *in vivo*.

In our BRET studies, we considered the possibility that the fused complemented luciferase might interfere with $G_{\alpha q/11}$ coupling to the heteromer. However, $G_{\alpha s}$, $G_{\alpha i}$ and $G_{\alpha o}$ coupling to the complemented receptors was as robust and consistent as with the individual protomers that were not complemented[5], and $G_{\alpha q/11}$ were robustly recruited to complemented receptors known to couple to these G proteins. Nonetheless, we also pursued studies with $G_{\alpha q/11}$ biosensors with DA receptors with unmodified cytoplasmic tails to rule out any potential disruption of $G_{\alpha q/11}$ recruitment due to the RLuc8 fusions. Although we observed robust activation of the $G_{\alpha s}$, $G_{\alpha i}$, and $G_{\alpha o}$ sensors, we again failed to observe evidence for $G_{\alpha q}$ or $G_{\alpha 11}$ activation by D1 and D2 receptors, individually or together, despite the fact that the $G_{\alpha q}$ and $G_{\alpha 11}$ sensors worked as expected for the M1 or 5HT2A receptors, both of which are known to couple to $G_{\alpha q}$. We conclude that DA receptors in any combination are unable to activate $G_{\alpha q/11}$ in HEK293 cells.

It is important to note that most of the published findings that led to the inference of $G_{\alpha q}$ as an intermediary mechanism for calcium mobilization did not study $G_{\alpha q}$ directly, but instead relied on calcium measurements or IP_3 production. While both of these are consistent with PLC activation, they do not necessarily require $G_{\alpha q/11}$ activation. Reports have shown GTPγS binding to $G_{\alpha q}$ upon activation of DA receptors but these methods depend on selective immunoprecipitation of endogenous $G_{\alpha q}$ by antibodies to differentiate the signal from that from other G_{α} isoforms[20, 25, 62, 63]. Using much more robust biosensors, we have failed to find evidence that D1 or D1/D2 receptors can activate $G_{\alpha q/11}$. Consistent with our results, Mailman and colleague also failed to detect IP3 release by D1, D2 or coexpressed receptors[23, 24]. Chun *et al*.[35] have recently shown in HEK cells that activation of coexpressed D1 and D2 receptors by dopamine leads to calcium mobilization, but this was blocked by a scavenger of $G_{\beta\gamma}$ as well as by pertussis toxin or cholera toxin, suggesting that the calcium signal results from downstream crosstalk and/or $G_{\beta\gamma}$-enhanced PLC signaling and not from direct activation of $G_{\alpha q}$ by DA receptors. We failed to observe calcium mobilization in response to 10 μM DA or SKF83959 in cells expressing D1, D2 or D1/D2 receptors (Fig. 3). We did observe a small calcium signal in response to both DA and SKF81297 at 100 μM (data not shown) in cells expressing D1 as well as both D1/D2 receptors. This suggests a minimal ability to engage PLC at extremely high concentrations of agonist, but this is clearly not heteromer-mediated since it occurs in cells expressing only D1, and is also not mediated by $G_{\alpha q/11}$, as demonstrated above.

SKF83959-induced behavioral responses were absent in D1 receptor knockout mice, confirming the role of the DA D1 receptor in mediating the motor effects of SKF83959. Additionally, we were able to block the effects of SKF83959 with the D1-like antagonist SCH23390 (data not shown). Since SKF83959 also has affinity for D5 receptors[35], we also assessed the SKF83959-induced behaviors in D5 receptor null mice. SKF83959-induced locomotor activity and grooming were largely intact in D5 receptor knockouts, further confirming the role of the D1 receptor as the primary D1-like receptor target for the motor-

activating effect of SKF83959. A small but statistically significant reduction of locomotion but not grooming responses in D5 receptor knockouts, however, suggests a possible accessory role for the D5 receptor in the actions of SKF83959.

Interestingly, we observed pronounced effects of SKF83959 in both D2 receptor and $G_{\alpha q}$ knockout mice, demonstrating that these proteins are not necessary for SKF83959-induced behavioral responses. These results, together with our signaling studies, contradict the current hypothesis in the literature regarding the signaling mechanism of SKF83959[10, 25]. In this model, SKF83959 acts through a D1/D2 receptor oligomer coupled to $G_{\alpha q}$ and a downstream signaling system involving PI hydrolysis and intracellular calcium release. If SKF83959 signaled through such a mechanism, then we would have expected to observe a loss of SKF83959-induced locomotor and grooming responses in the D2 receptor and $G_{\alpha q}$ null mice, as we did with the D1 receptor knockout mice. Contrary to this hypothesis, however, D2 receptor and $G_{\alpha q}$ knockout mice appear to be more sensitive to SKF83959; both exhibiting greater percentage change from baseline in the locomotor assay compared to WT mice (data not shown). Previous findings have demonstrated that SKF83959-induced behaviors can be blocked by the D2-like receptor antagonist raclopride[25, 26], a finding we have replicated (data not shown). However, these results are confounded by the fact that the doses required to block SKF83959-induced behaviors induces significant catalepsy[9, 64], suggesting, in light of our results with the D2 receptor knockout mice, that the inhibition of locomotion by raclopride is not mediated at a D1/D2 receptor heteromer but rather is indirect through blockade of D2 receptor signaling.

Previous reports have also suggested that Thr^{286} phosphorylation of CaMKIIα is a critical downstream component in the signaling of SKF83959[25, 26, 55]. However, we observed intact SKF83959-induced locomotor and grooming responses in CaMKIIα–Thr^{286}Ala knockin mice that lack the major regulatory autophosphorylation site, suggesting again that CaMKIIα signaling is not an essential component of SKF83959-induced signaling.

Our data also directly challenge the idea that D1 and D2 receptors form oligomers in medium spiny neurons (MSNs) in adult animals. Co-expression of D1 and D2 receptors in MSNs has been a matter of long debate[6]. Whereas *in situ* hybridization[7] supports an almost complete separation of D1- and D2 receptor-expressing MSNs, single-cell PCR[65] and immunohistochemical[8–12] methods showed a larger degree of colocalization. The use of BAC transgenic reporter mice confirmed that the segregation of the two receptors is not complete[6, 66], particularly in the shell of the nucleus accumbens[60]. Our data using the Drd1a-tdTomato/Drd2-eGFP double BAC transgenic mice directly confirm that a small number of neurons do express both D1 and D2 receptors, especially within the shell of the nucleus accumbens and the caudal tail of the neostriatum.

However, using traditional immunohistochemistry or PLA, we were unable to detect colocalization or molecular proximity of D1 and D2 receptors in the adult ventral striatum, in contrast to previous reports[8–10, 28]. This discrepancy is not readily explained by differences in anti-D2 receptor primary antibodies or immunostaining protocols used, since we also failed to detect colocalization using antibodies and conditions identical to those published (data not shown). The lack of PLA signal is unlikely to be related to a lack of

sensitivity of the assay, since we have succeeded in detecting endogenous receptor complexes in the striatum, including D2/A2A receptors[47] or D1/NR1 receptors[67] (see also Biezonski et al., in revision). Moreover, we failed to detect colocalization of D1 and D2 receptors by immunohistochemistry in 3 different mammalian species (i.e. mouse, rat and monkey). It is important to note that when colocalization and interaction between D1 and D2 receptors was described previously, it was most evident within cell bodies[8–10, 28]. However, DA receptors have generally been shown to be mainly expressed in the neuropil[11, 46, 47, 59, 61], even when overexpressed[46, 47]. Moreover, we were able to show that, even when one or the other receptor was overexpressed, the level of colocalization/molecular proximity of D1 and D2 receptors was extremely low, suggesting that an active mechanism may keep the receptors segregated at the subcellular level. Importantly, our data are in accordance with the observation using electron microscopy that D1 and D2 receptors do not colocalize at the subcellular level even when expressed in the same cellular compartments[59]. The mechanisms that support segregation of those two receptors are unknown but could rely on distinct properties of the individual receptors, such as interaction with specific scaffolding proteins or localization in different membrane microdomains. Nonetheless, our data in HEK cells ([5]; current study) as well as *ex vivo* with overexpression of both D1 and D2 suggest that the receptors have the ability to interact under conditions in which the segregating mechanism is either not present or is overwhelmed by receptor excess.

Despite the fact that our data do not support the existence of D1/D2 receptor heteromers in adult brain, the presence of cells within the shell of the nucleus accumbens that co-express both receptors does suggest the existence of a third neuronal circuit within the basal ganglia, distinct from the classical direct and indirect pathways of the striatum, that could exhibit atypical signaling and physiological properties[10]. It is conceivable that the extent of D1 and D2 receptor coexpression and/or heteromerization in these cells varies during different developmental periods (although heteromerization of these receptors was reported to be less, not more, in juvenile brain[68]), or even in pathological states that might alter the segregation process that normally keeps the receptors apart. Regardless, a systematic characterization of the anatomical, physiological and molecular properties of the small subset of neurons that coexpress D1 and D2 receptors will be necessary to understand the role of these cells in basal ganglia physiology and pathophysiology[6, 60].

Our current data call into question the interpretations of several previous studies suggesting the presence of a D1/D2 heteromer-containing neurons as a target for drug discovery (for review, see[10, 69]). In fact, dysregulation of D1/D2 receptor heteromers has been specifically postulated in both schizophrenia[9] and depression[27]. The main findings relevant to schizophrenia have been increases in the high affinity state of D2 receptors that can be preferentially competed with SKF83959 in the striatum following repeated amphetamine and in the post-mortem globus pallidus of schizophrenia patients[9]. For major depression, a peptide that disrupts D1/D2 co-immunoprecipitation produced anti-depressant effects in an animal model[27]. While our findings cannot rule out the possibility of increased D1/D2 interaction in pathological states, our complete inability to support this interaction in WT mice, rats and monkeys suggests that a role for D1/D2 heteromers in pathological conditions

must be reconsidered. Moreover, our findings challenge the idea that D1/D2 heteromers are promising targets for the development of highly selective ligands for treatment of psychiatric symptoms. Rather than focusing on D1/D2 heteromer ligands, basic and clinical research programs might gain more traction by instead performing mechanistic evaluation of D1 receptor agonists, such as DAR-0100A, which was recently shown to attenuate working memory impairments in patients with schizotypal personality disorder[70].

Taken together, these data suggest that current models of DA receptor functional selectivity based on D1/D2 heteromerization are incorrect, and that D1/D2 heteromers do not play a significant role in the normal adult brain. The field needs to reconsider previous data interpreted with this hypothesis in mind.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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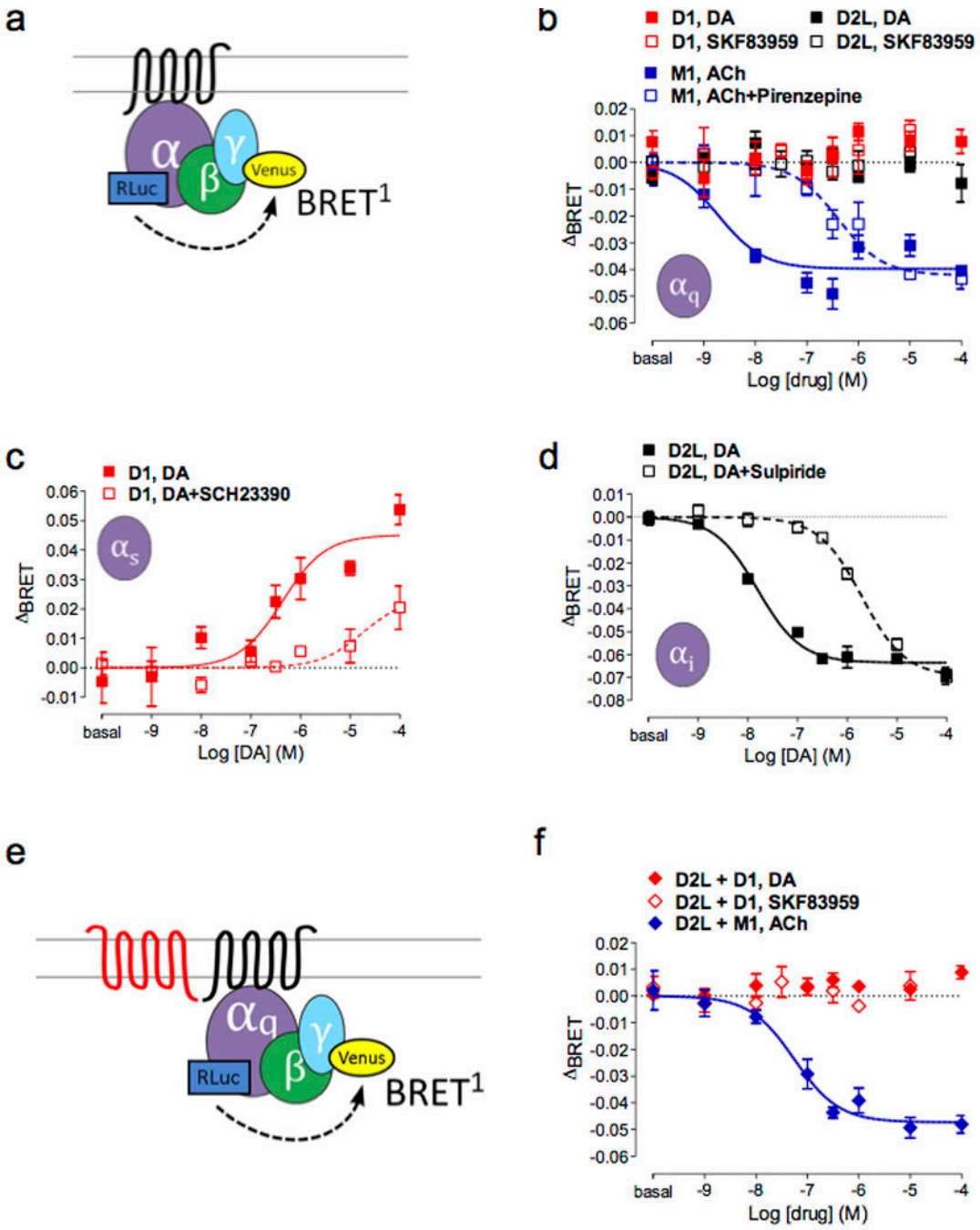

Figure 1.

Drug-induced conformational change of $G_{\alpha q}$ is not detected in D1 and/or D2L-expressing cells. (**a**) Drug-induced BRET change shown as ΔBRET (= BRET ratio with drug applied - basal BRET ratio) is detected between G_{α} and G_{γ} subunits. Note that due to the different positions of the donor sensors within the G_{α} subunits, activation of the heterotrimer is manifested as a decrease in BRET between the G_{α} donor and the G_{γ} acceptor for the $G_{\alpha q}$ and $G_{\alpha i1}$ sensors but as an increase in BRET for the $G_{\alpha s}$ sensor. (**b**) M1R is shown as a positive control for $G_{\alpha q}$ activation (blue solid = ACh, blue open = ACh + pirenzepine, $p <$

0.001) whereas $G_{\alpha q}$ activation was not observed for either DA or SKF83959 in the D1R (red) or D2R (black). Conformational change of cognate G proteins by DA was induced and blocked by an agonist and an antagonist for (**c**) D1R (red, $p < 0.001$), (**d**) D2LR (black, $p < 0.001$). (**e**) Diagram showing the BRET configuration in (f). (**f**) Co-expression of D1 and D2L did not lead to $G_{\alpha q}$ activation after drug stimulation (red solid = DA, red open = SKF83959) whereas robust $G_{\alpha q}$ coupling to M1R was still seen after acetylcholine addition in the presence of D2LR (blue, $p < 0.001$).

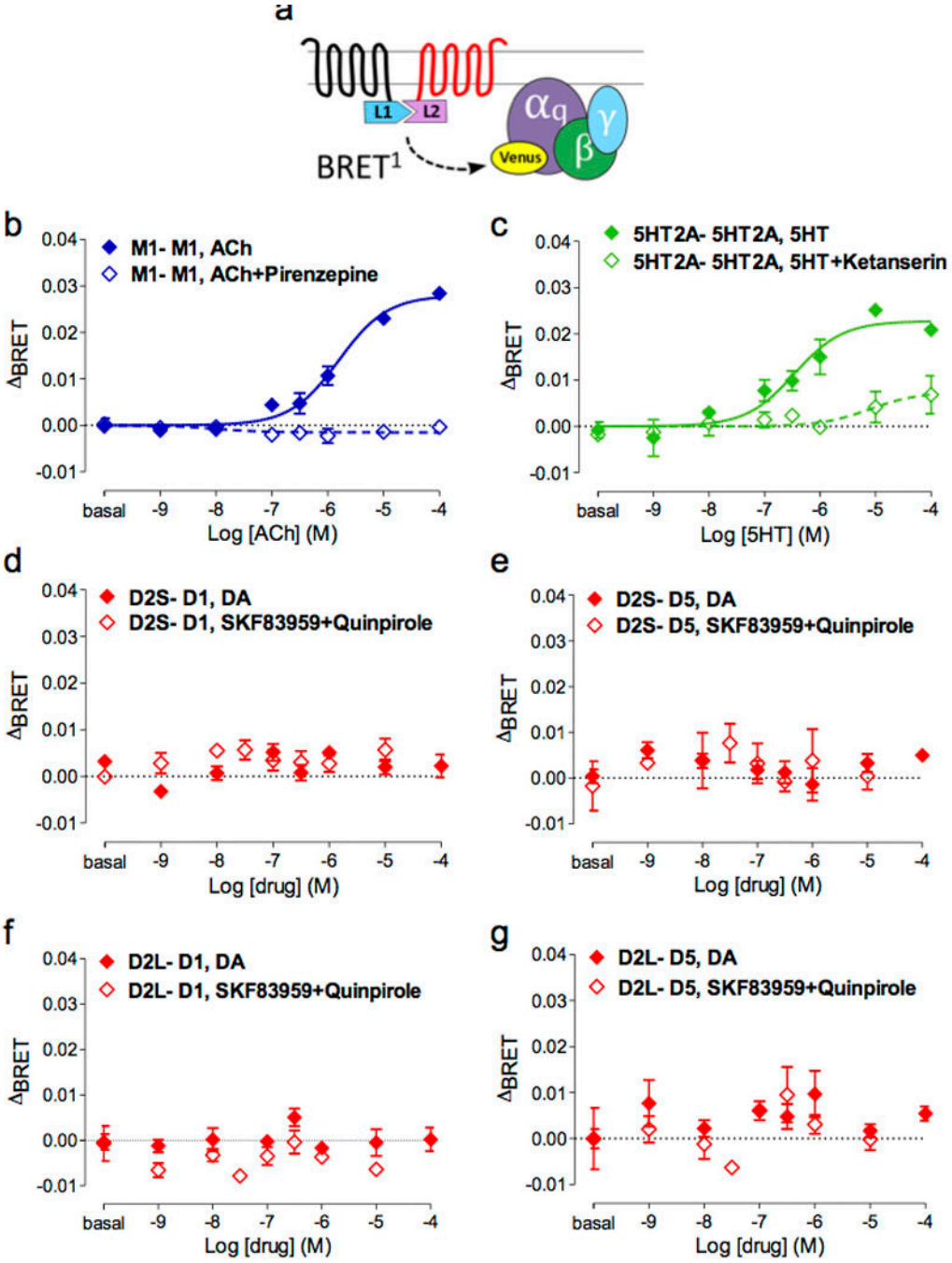

Figure 2.

Defined receptor dimer pairs do not recruit $G_{\alpha q}$ after drug stimulation. Using the CODA-RET approach, a receptor dimer pair is defined by complementation of the luminescent protein Rluc8. (**a**) Diagram showing the BRET configuration. (**b, c**) The complemented M1RL1-M1RL2 and 5HT2ARL1-5HT2ARL2 pairs are shown as positive controls for $G_{\alpha q}$ coupling (solid = agonist, open = agonist + antagonist, $p < 0.001$). In contrast, the complemented DA receptor dimer pairs shown (**d**) D2SR-D1R, (**e**) D2SR-D5R, (**f**) D2LR-

D1R, (**g**) D2LR-D5R] failed to reveal drug-induced $G_{\alpha q}$ recruitment (red solid = DA, red open = SKF83959 + quinpirole).

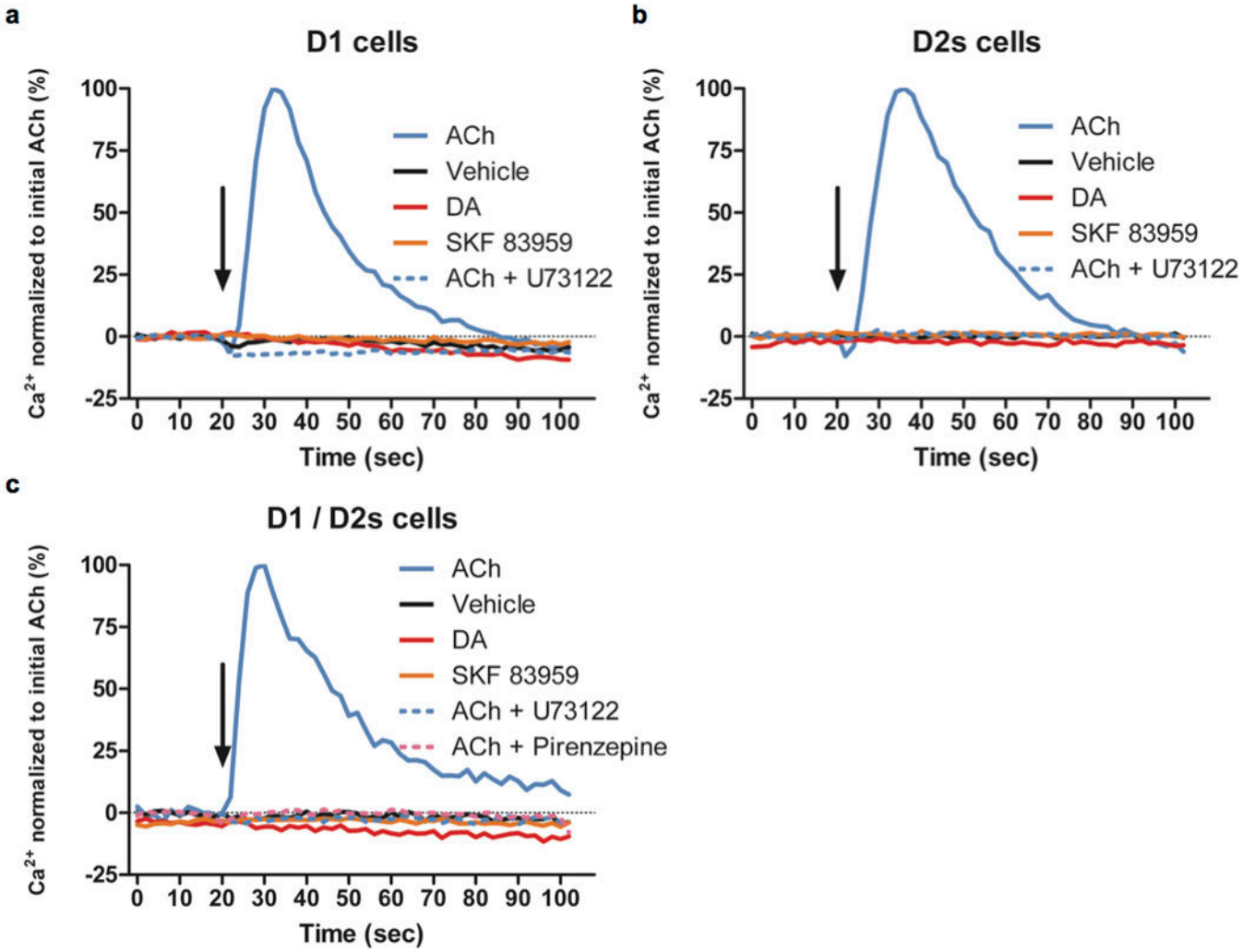

Figure 3.

Neither DA nor SKF 83959 induces calcium mobilization in stable cell lines expressing D1 and/or D2 receptors. Using the FLIPR5 calcium assay system, intracellular calcium levels were measured every 2 seconds and plotted against time. Ligands (ACh = 10 μM acetylcholine, vehicle, DA = 10 μM dopamine, 10 μM SKF83959, 10 μM U73122, or 10 μM pirenzepine) were added at 20 secs, indicated by an arrow, in (**a**) D1R, (**b**) D2R, and (**c**) D1R/D2R stable cells. Calcium level is shown in percentage normalized to 10 μM acetylcholine (ACh). Traces are representatives of n = 3 experiments.

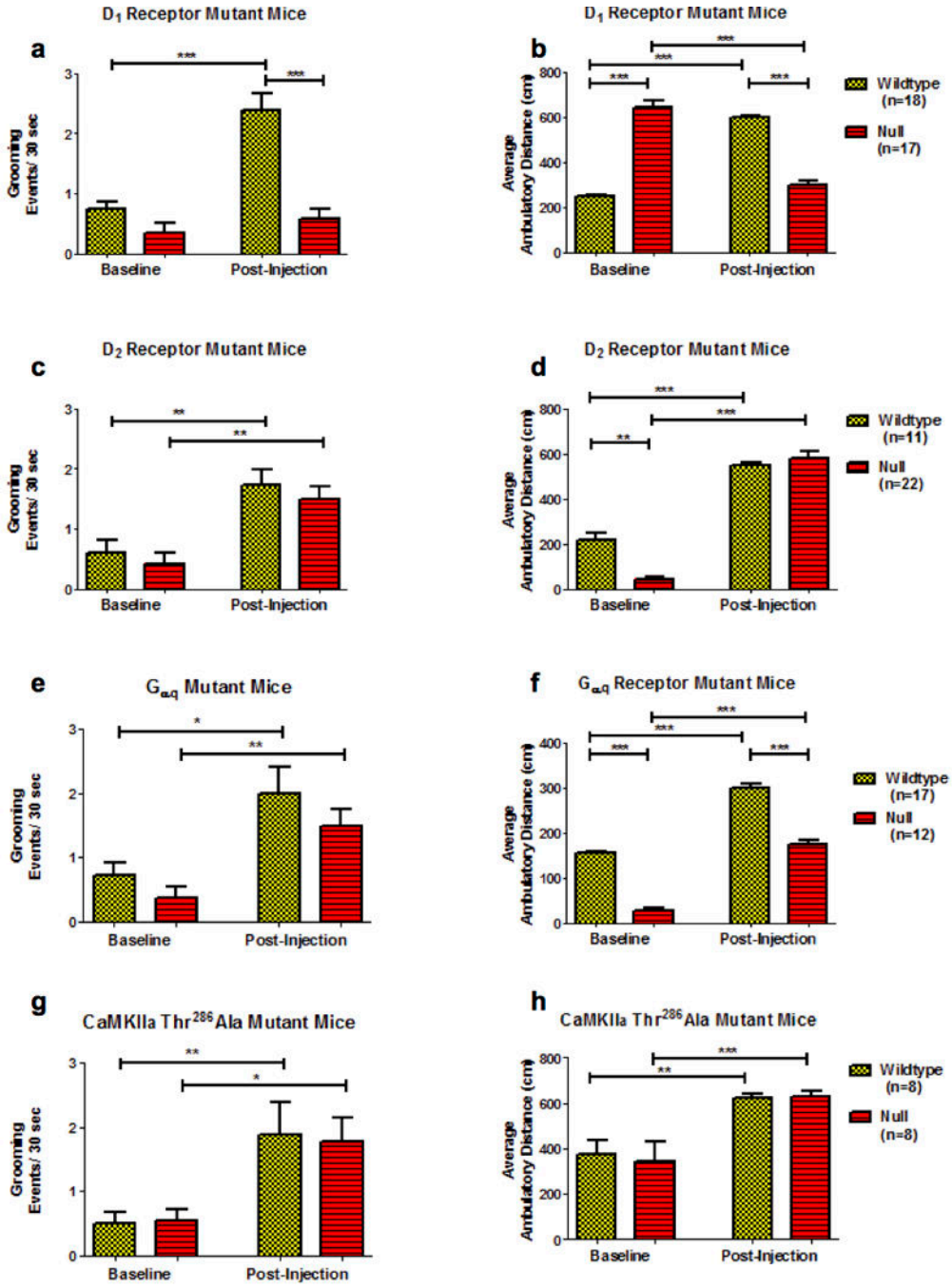

Figure 4.

SKF83959-induced orofacial grooming (a, c, e, g) and locomotor activation (b, d, f, h) is absent in mice lacking the D1 receptor (a, b) but present in D2 receptor knockouts (c, d), Gαq null (e, f) and CaMKII (g, h) mutant mice. Asterisks denote significance (*p<0.05, **p<0.01, ***p<0.001) based on *post-hoc* Bonferroni multiple comparison tests following repeated measures ANOVA. The data contained in panel f from the Gαq null mice are a modified presentation of those published in[42]. Yellow bars = WT, Red bars = Mutant.

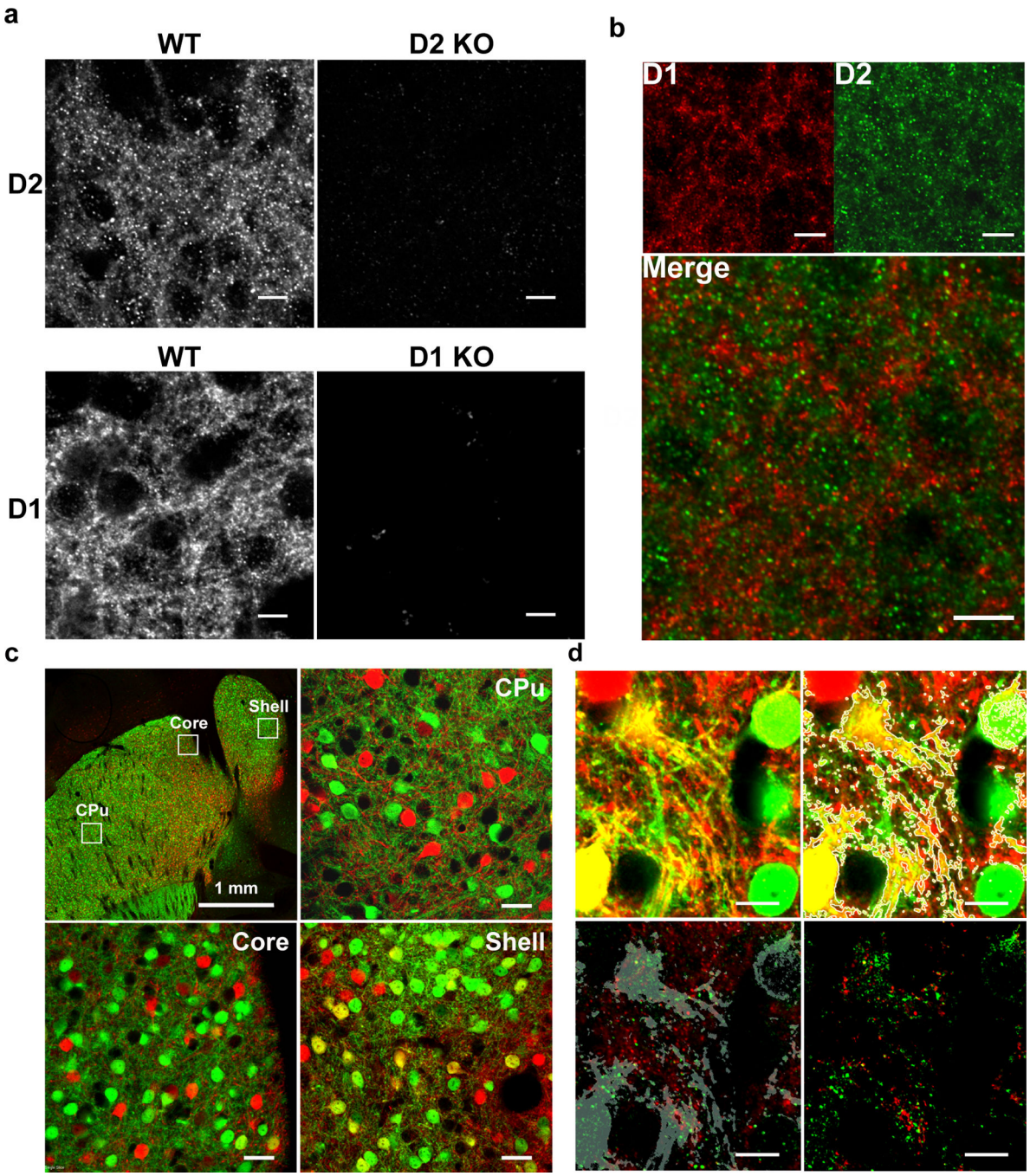

Figure 5.

D1 and D2 receptors are co-expressed but do not colocalize in the shell of the nucleus accumbens. (a) Immunohistochemical detection of D2 (top) and D1 (bottom) in the shell of the nucleus accumbens in WT mice. Staining was virtually absent in D2 or D1 KO mice. (b) Co-staining of D1 and D2 receptors in the shell of the nucleus accumbens in mice revealed extremely limited colocalization. (c) Top-left: Low magnification confocal image of a horizontal section going through the striatum and nucleus accumbens in a double BAC Drd1a-tdTomato/Drd2-eGFP transgenic mouse. The Ds-Red (red) and eGFP (green) signals

were enhanced using specific antibodies. Cells co-expressing active *Drd1* and *Drd2* promoters (yellow) were detectable in the shell (bottom-right) but not in the core (bottom-left) of the nucleus accumbens or in the dorsal striatum (CPu: top-right). (d) Zoom on tomato+ (D1) and eGFP+ (D2) dendritic areas in the shell of the nucleus accumbens after quadruple staining of DsRed, eGFP, D1 and D2. Top-left: tomato and eGFP signals; Top-right: yellow areas were detected using ImageJ software and identified with a mask (grey outline); Bottom-left: D1 (green) and D2 (red) receptors signals merged with the mask (grey). Bottom-right: D1 (red) and D2 (green) signals extracted within the mask area only showed no colocalization of D1 and D2 receptors. Scale bars=10 μm unless otherwise indicated.

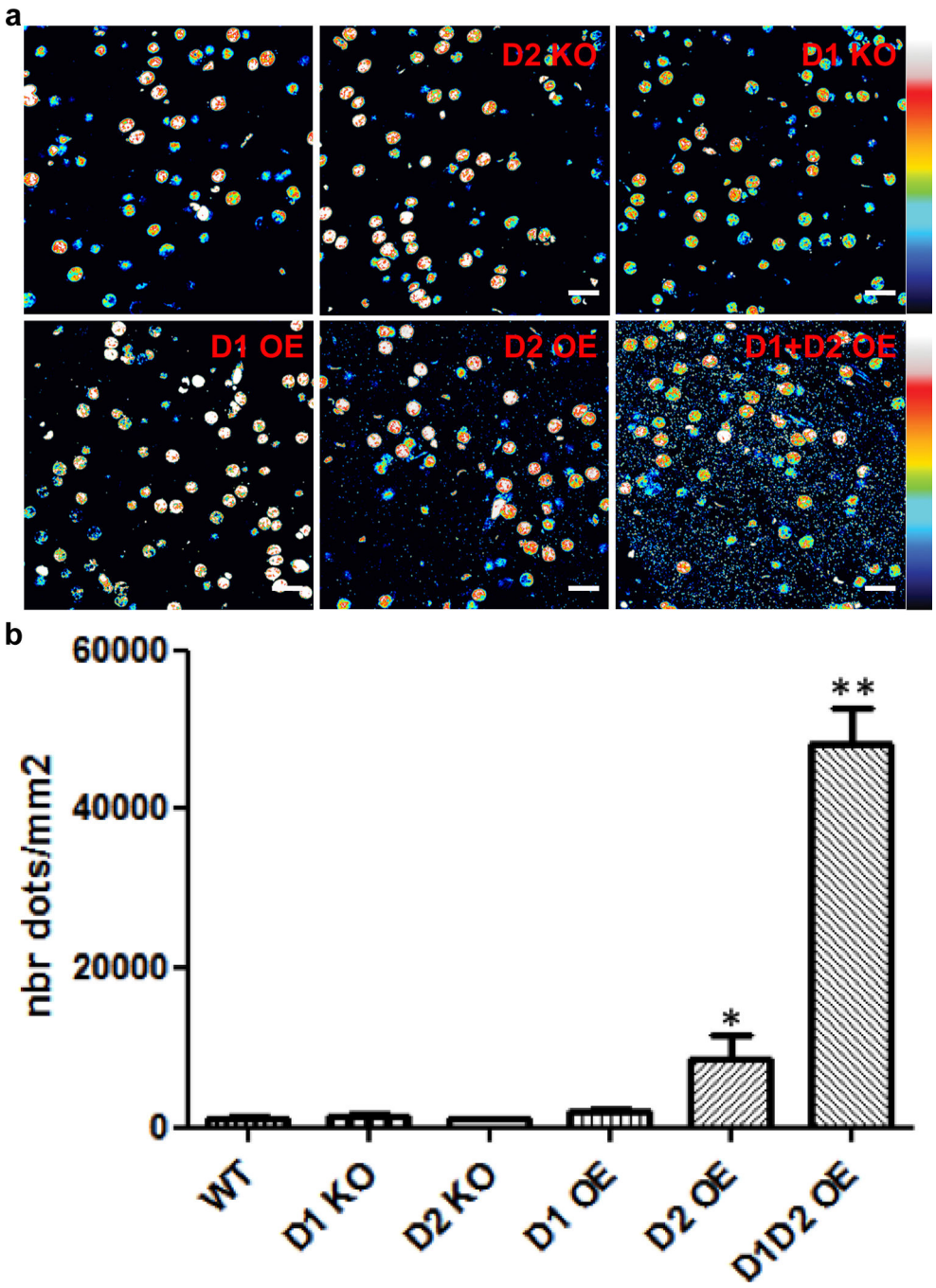

Figure 6.

Proximity ligation assay (PLA) shows that endogenous D1 and D2 receptors do not physically interact in the shell of the nucleus accumbens. (a) Whereas the non-specific nuclear background did not vary between conditions, the PLA signal (small dots) indicative of molecular proximity between D1 and D2 receptors was virtually absent in WT mice, as well as in D1 and D2 KO mice. Virally-mediated overexpression of D1 (D1OE) did not significantly increase PLA signal, whereas D2 (D2OE) receptor overexpression led to a small, but significant increase in PLA signal. Simultaneous overexpression of both receptors

(D1D2OE) led to a very strong PLA signal, suggesting that the 2 receptors have the ability to interact if highly expressed in close proximity. (b) Quantification of the PLA signals was performed as described in Methods. N=4–8 (3–4 animals). * $p<0.05$; ** $p<0.01$. Scale bars=10 μm.