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D₅ Dopamine Receptors are Required for Dopaminergic Activation of Phospholipase C

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ABSTRACT

Dopamine activates phospholipase C in discrete regions of the mammalian brain, and this action is thought to be mediated through a D₁-like receptor. While multiple lines of evidence exclude a role for the D₁ subtype of D₁-like receptors in the phosphoinositide response, the D₅ subtype has not been similarly examined. Here, mice lacking D₅ dopamine receptors were tested for dopamine agonist-induced phosphoinositide signaling both in vitro and in vivo. The results show that hippocampal, cortical and striatal tissues of D₅ receptor knockout mice significantly or completely lost the ability to produce inositol phosphate or diacylglycerol messengers following stimulation with dopamine or several selective D₁-like receptor agonists. Moreover, endogenous inositol-1,4,5-trisphosphate stimulation by the phospholipase C-selective D₁-like agonist, SKF83959, was robust in wildtype animals but undetectable in the D₅ receptor mutants. Hence, D₅ receptors are required for dopamine and selective D₁-like agonists to induce phospholipase C-mediated phosphoinositide signaling in the mammalian brain.

Introduction

Multiple subtypes of dopamine receptors expressed in the mammalian brain may be categorized into D₁-like and D₂-like subclasses based on their structural homology, pharmacological selectivity, and functional similarities (Civelli et al., 1993; Lachowicz and Sibley, 1997). Members of the D₁-like subclass include the D₁ and D₅ receptors (Civelli et al., 1993; Lachowicz and Sibley, 1997). Expressed in clonal cell lines, both the D₁ and D₅ receptors have demonstrated coupling to multiple signaling cascades as assessed through the activation of specific G proteins or the formation of downstream second messengers (Sidhu and Niznik, 2000; Neve et al., 2004). However, promiscuous or inconsistent coupling to multiple or alternate G proteins and signaling cascades might be a frequent feature for many G protein-coupled receptors expressed in artificial cell lines (Sidhu and Niznik, 2000; Hermans, 2003). Thus, experiments aiming to definitively assign a signaling pathway to a given receptor would benefit from the use of physiological tissue preparations and receptor-selective pharmacological agents.

Currently, while various chemical entities that are highly selective for D₁-like receptors exist, there is still a lack of agonists or antagonists that sufficiently discriminate between the D₁ and D₅ subtypes. Moreover, typical agonists of D₁-like receptors generally activate both the D₁ and D₅ subtypes, and elicit multiple signaling responses in mammalian tissues (Neve et al., 2004). Such responses include cascades mediated by adenylyl cyclase (Monsma, Jr. et al., 1990), MAP kinase (Zhen et al., 1998), intracellular calcium mobilization (Lezcano and Bergson, 2002; Yasumoto et al., 2004), and phospholipase C (PLC) (Felder et al., 1989; Undie and Friedman, 1990). The PLC response, which is associated with the hydrolysis of phosphatidylinositides (PIs)

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to release inositol phosphate and diacylglycerol second messengers, is evident in discrete brain regions, including tissues expressing D₁-like receptors that show relatively negligible functional coupling to adenylyl cyclase (Undie and Friedman, 1990; Montague et al., 2001), and persists when D₁ receptors have been genetically deleted (Friedman et al., 1997) or chemically inactivated (Rosengarten and Friedhoff, 1998; Undie et al., 2000). Thus, although a D₁-like receptor mediates dopaminergic-induced PI signaling in the brain, the structurally defined D₁ receptor itself is not the specific subtype that is involved. Given that extensive data mining of mammalian genomes has not revealed the existence of additional dopamine receptors beyond the D₁-D₅ entities, the foregoing suggests that the D₅ subtype might be the D₁-like receptor that mediates the PLC response. This hypothesis has not been directly tested in the intact brain.

In the present work, we have sought to further examine the nature of D₅ receptor coupling in native brain tissues. For this, we used mutant mice lacking D₅ receptors to test the hypothesis that the D₅ receptor is required for dopaminergic activation of PLC in the intact brain. The results demonstrate that dopamine and selective D₁-like receptor agonists lose their efficacy to induce inositol phosphate accumulation or diacylglycerol production in brain tissues of mice lacking the D₅ subtype of D₁-like receptors.

Materials and Methods

Animals. Dopamine D₅ receptor knockout (D₅KO) mice were obtained along with their wildtype littermates from the National Institutes of Health. Produced from a genetic background of 129/SvJ1 and C57BL/6J, the mice are viable, develop normally, and are fertile and capable of reproduction (Hollon et al., 2002). The genotype of each animal was determined by PCR techniques, and confirmed D₅KO animals showed complete loss of immunoreactivity for the D₅ receptor in the brain (Hollon et al., 2002). Mice used in this study were at least 10 weeks old. Protocols for the care and use of the animals were approved by the Institutional Animal Care and Use Committee and conformed to the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*.

Drugs and chemicals. The D₁-like agonists SKF38393 (1-phenyl-2,3,4,5-tetrahydro-(H1)-3-benzazepine-7,8-diol) and SKF83959 (3-methyl-6-chloro-7,8-dihydroxy-1-[3methylphenyl]-2,3,4,5-tetrahydro-1H-3-benzazepine) were obtained from the NIMH Chemical Synthesis Program (NIMH, Bethesda, MD). Dopamine and the buffer reagents were purchased from Sigma-Aldrich (St. Louis, MO). Drugs were dissolved in saline and diluted into HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) bicarbonate (HB) buffer (Undie and Friedman, 1990). Each experiment was performed on multiple occasions, each time using fresh preparations of drugs. Protein was assayed by the Bradford method using reagents from BioRad (Hercules, CA).

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Assay of phosphoinositide hydrolysis in brain slices. Brain slices were prepared from wildtype and D₅KO mice as previously described (Undie and Friedman, 1992). Tissue slices were preincubated for 45 min in calcium-free HB buffer (Undie and Friedman, 1992), and then distributed in 25- μ l aliquots (300 μ g protein) to 5-ml polypropylene tubes containing 150 μ l of normal HB buffer (contains 1.2 mM CaCl₂). The assay tubes were aerated with 95% O₂/5% CO₂ and incubated at 37 °C in a shaking water bath. Tissues were double-labeled with [³H]inositol (6 μ Ci/ml) and [¹⁴C]cytidine (0.6 μ Ci/ml) to respectively monitor the activities of inositol phosphates and the diacylglycerol metabolite CDP-diacylglycerol (Undie, 1999). Following 30 min of the labeling reaction, 5 mM LiCl was added, followed 5 min later by addition of indicated concentrations of dopamine or a D₁-like agonist. HB buffer was added as necessary to give a final incubation volume of 250 μ l and incubation continued for an additional 60 min. The reaction was stopped by mixing the slices with 1.5 ml of chloroform:methanol:1 M HCl (100:200:1). After incubating the mixture at room temperature for 45 min, followed by vigorous vortexing to complete the extraction process, the inositol phosphates and unreacted [³H]inositol or [¹⁴C]cytidine were partitioned from the phospholipids by sequential mixing with 0.5 ml chloroform and 0.75 ml deionized water. The mixture was vortexed for 3 min and then centrifuged at 1000 x g for 5 min. A 1-ml aliquot of the aqueous phase was used to assay the content of inositol phosphate species by Dowex anion exchange chromatography (Undie and Friedman, 1990). Chromatographic eluates were mixed with 8 ml of Scintisafe gel (Fisher Scientific, Pittsburgh, PA) to convert the samples into gel form and the tritium radioactivity was counted by liquid scintillation spectrometry. A 400- μ l aliquot of the organic phase was quantitatively transferred into scintillation vials, allowed to dry overnight at room temperature,

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redissolved in 5 ml of Biosafe scintillation cocktail, and the tritium ($[^3\text{H}]$ phosphoinositides) and C-14 ($[^{14}\text{C}]$ CDP-diacylglycerol) activities concurrently measured using the Beckman LS6500 spectrometer. Data for each analyte were converted to dpm/ μg protein as previously described (Undie and Friedman, 1990; Undie, 1999).

Assay of endogenous brain phosphoinositide hydrolysis. Agonist-induced release of endogenous inositol-1,4,5-trisphosphate was assayed *ex vivo* following in vivo drug treatments and subsequent excision of requisite test tissues. Mice were injected subcutaneously with saline 5 ml/kg (controls) or with selected dosages of test agents dissolved in 5 ml/kg saline. After allowing 15 min for the drug to act, the animals were rapidly decapitated and the brains removed and washed in ice-cold HB buffer. Brain regions of interest were dissected out, transferred into 2-ml Eppendorf tubes and rapidly frozen in liquid nitrogen. If the tissues were not to be processed immediately, they were stored at $-70\text{ }^{\circ}\text{C}$ until use. Frozen tissues were thawed to $4\text{ }^{\circ}\text{C}$ and homogenized in 1 ml of ice-cold 1M trichloroacetic acid using a Polytron homogenizer set at medium speed for 15 s. The tubes were placed on ice for 15 min, and then centrifuged at 13,000g for 10 min at $4\text{ }^{\circ}\text{C}$. The supernatant containing extracted inositol trisphosphate was transferred to another Eppendorf tube. The pellet was washed three times with distilled water, digested in 1M NaOH, and the mixture used for protein determination. To 0.5 ml of the supernatant was added 1ml of a mixture of trichlorotrifluoroethane-trioctylamine (3:1), mixed vigorously for 15 s, and centrifuged for 1 min at 10,000 g. An aliquot of the upper phase was used for inositol-1,4,5-trisphosphate determination by the radioreceptor binding assay using reagents and protocols

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from Amersham (Piscataway, NJ, USA) (Cook et al., 1990). Inositol-1,4,5-trisphosphate content in the tissue samples is expressed in pmol/mg tissue protein.

Data analysis. Data for each brain region were normalized across experimental runs and then subjected to two-way analysis of variance (ANOVA) of second messenger response by drug concentration by phenotype. Upon detecting statistical significance in the ANOVA results, *posthoc* Bonferoni analyses were performed to compare pairs of D₅KO and WT responses at each drug concentration (or *in vivo* dose). To determine if any of the drug concentrations or doses exerted significant effects relative to baseline, *posthoc* Dunnett tests were performed. Statistical comparisons were considered significant at $p < 0.05$ or better.

Results

Effects of the D₅KO phenotype on dopamine agonist stimulation of phosphoinositide

hydrolysis in brain slice preparations. Using a range of drug concentrations known to elicit dopaminergic stimulation of PI hydrolysis, we tested the effects of dopamine and selective D₁-like agonists in brain slice preparations of WT and D₅KO mice. The data are shown in Fig. 1. Two-way ANOVA analyses (inositol phosphate response by phenotype by drug concentration) for each drug indicated highly significant overall effects of concentration ($p < 0.001$) and phenotype ($p < 0.001$) on the second messenger responses. Subsequent Bonferoni tests revealed that basal accumulation of inositol phosphate was not significantly different between wildtype and mutant frontal cortex or striatum, but was significantly lower in the hippocampus ($p < 0.01$) – the brain region of highest D₅ receptor expression (Montague et al., 2001).

Dopamine at the maximally effective concentrations of 100-300 μ M (Undie and Friedman, 1990) induced significant accumulations of inositol phosphate in brain slice preparations of wildtype mice ($p < 0.001$ for each tissue), and in the frontal cortex of mutant mice ($p < 0.01$). In each test tissue, however, drug effects in the mutant group were significantly reduced compared to the wildtype group ($p < 0.0001$; ANOVA main effects of phenotype for each drug and tissue type). Effects of the selective D₁-like receptor partial agonist, SKF38393, were significant and concentration-dependent, as would be expected, in the wildtype group; in the mutant group, however, the main ANOVA performed on only the mutant tissue data did not show any significant concentration-related effects of SKF38393 for any of the test tissues. With

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SKF83959, a selective D₁-like agonist that does not stimulate adenylyl cyclase but potently stimulates PI hydrolysis both *in vitro* and *in vivo* (Gnanalingham et al., 1995; Panchalingam and Undie, 2001; Jin et al., 2003), significant and concentration-related effects (up to 10 μM) were obtained in each tissue of wildtype animals (p<0.001 for each tissue) but not in any of the tissues of the mutant group.

To test the diacylglycerol arm of the PI cycle, test tissue slices were concurrently labeled with [¹⁴C]cytidine so that newly formed diacylglycerol may be converted to [¹⁴C]CDP-diacylglycerol and thereby radiometrically detected (Undie, 1999). As shown in Fig. 2, tissues from wildtype mice responded to dopamine or the selective D₁-like agonists in a concentration-related manner, whereas the tissues from the mutant animals showed markedly reduced or absent effects. When data from the mutant tissues were isolated from the wildtype data and analyzed, there were no concentration-related effects for any of the drugs in any of the tissues except for the effects of dopamine in the mutant striatal tissues where the main ANOVA was significant (p<0.01) and the 300 μM concentration differed significantly from control (p<0.01). Again, there were no differences in basal accumulations of CDP-diacylglycerol among the phenotypes, with the exception of the hippocampus where loss of functional D₅ receptors was associated with a statistically significant decrease (p<0.05) in basal levels of the product. Overall, the [¹⁴C]CDP-diacylglycerol data (Fig. 2) were in agreement with the [³H]inositol phosphate data (Fig. 1). Hence, the knockout phenotype markedly decreases or completely abolishes the ability of different D₁-like receptor-effective agonists to induce brain PI hydrolysis.

To test if the effects of the D₅-knockout phenotype on PLC signaling were specific to the dopamine system, we examined the effects of the selective α-adrenergic receptor agonist,

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phenylephrine, on inositol phosphate and CDP-diacylglycerol accumulation in frontal cortex tissues. As shown in Fig. 3, phenylephrine significantly stimulated inositol phosphate and CDP-diacylglycerol in both the wildtype and the mutant tissues ($p < 0.001$ for each analyte in each phenotype). Bonferoni posthoc comparisons revealed no significant effect of phenotype on basal or phenylephrine-induced accumulation of inositol phosphate or diacylglycerol messengers.

Effects of SKF83959 on in vivo release of inositol-1,4,5-trisphosphate. In anticipation of a physiological relevance for the phenotypic differences in signaling efficacy, we tested the ability of a brain-accessible D_1 -like agonist to induce second messenger production in the brains of intact animals. Mice were administered behaviorally relevant dosages of the PLC-selective D_1 -like agonist, SKF83959 (Panchalingam and Undie, 2001; Jin et al., 2003). Endogenously formed inositol-1,4,5-trisphosphate levels were then measured in discrete brain tissues of wildtype and D_5 mutant mice and the results are shown in Fig. 4. Basal levels of inositol-1,4,5-trisphosphate were between 65 and 85 pmol/mg protein in the test tissues; these values are within range of previous reports (Dwivedi et al., 2000; Jin et al., 2003). There were no significant differences in basal levels compared between wildtype and mutant animals in either the striatum or the hippocampus. SKF83959 induced significant and dose-dependent increases in inositol-1,4,5-trisphosphate levels in both the striatum and hippocampus of wildtype mice. The mutant animals, however, failed to show any significant increases in inositol-1,4,5-trisphosphate levels in response to the drug treatment. Apparently, the presence of D_5 receptors is required for SKF83959 to activate PLC-mediated inositol second messenger formation in the intact brain.

Discussion

Dopaminergic stimulation of PI signaling has long been known to be mediated through a D₁-like receptor mechanism (Felder et al., 1989; Undie and Friedman, 1990). Related studies have excluded a role for the cloned D₁ receptor (Undie et al., 1994; Friedman et al., 1997), leaving the possibility that the D₅ receptor may be the subtype that mediates the PLC response. But this notion has never before been directly tested. The present findings show that the D₅ receptor is indeed crucial in the mediation of dopamine's stimulatory effects on PI metabolism. This inference is consistent with known characteristics of dopamine-induced PI signaling and D₅ receptor expression among the brain regions. For instance, as indicated in past and the present results, both receptor expression and agonist-induced signaling are relatively higher in the hippocampus than in the striatum, with intermediate effects in the prefrontal cortex (Undie and Friedman, 1990). Other consistent observations are those indicating that the D₅ receptor can couple to G_q-like G proteins in various cell lines or in renal brush border membranes (Sidhu and Niznik, 2000), that the receptor directly modulates calcium currents and burst firing in the subthalamic nucleus (Baufreton et al., 2003), and it frequently exists in extrasynaptic microdomains associated with neuronal inositol-1,4,5-trisphosphate-sensitive calcium stores (Paspalas and Goldman-Rakic, 2004). The present findings, therefore, could provide new insights toward understanding those agonistic D₁-like effects that have hitherto defied explanation on the basis of adenylyl cyclase coupling.

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Our current results are also supported by previous experiments involving the expression of a D₁-like receptor encoded by striatal mRNA in *Xenopus* oocytes. Mahan *et al.* (Mahan et al., 1990) found that injection of rat striatal mRNA into *Xenopus* oocytes led to the expression of a D₁-like receptor coupled to inositol phosphate production and Ca²⁺ mobilization. However, expression of the cloned rat D₁ receptor in the oocytes lead to the production of cAMP, but not Ca²⁺ mobilization, suggesting that the D₁ receptor was not linked to the PI response (Monsma, Jr. et al., 1990). Moreover, using size fractionation techniques, it was shown that mRNA encoding the striatal PI-linked D₁-like receptor was between 2.5-3.0 kb in size, thus distinguishing it from the 4.1 kb mRNA fragment that encodes the rat D₁ receptor. Interestingly, the rat D₅ receptor is encoded by an mRNA that is ~3 kb in size (Tiberi et al., 1991), in close agreement with the size of the mRNA encoding the PI-linked D₁-like receptor identified using the oocyte expression system. Our conclusion is that the Ca²⁺ response observed in these early oocyte expression experiments involved the D₅ receptor subtype.

Experiments with selective α -adrenergic receptor stimulation in frontal cortical tissues revealed that the effects of the D₅ receptor loss may be specific to the dopamine system rather than constitute a generalized deficit in response to PLC-coupled monoaminergic receptors. Moreover, there was general agreement between the in vitro and in vivo drug effects. Although baseline accumulations of inositol phosphate or CDP-diacylglycerol were lower in the mutant hippocampal slices compared to the wildtype tissue, no such differences were observed in the striatum or frontal cortex; nor were any hippocampal differences evident in the in vivo assay. Further, factoring out the basal accumulation for each phenotype did not change the outcome of

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the analyses of drug effects, thus indicating that the difference in drug effects among the phenotypes outweighed any baseline differences.

For the *in vivo* studies, it was particularly noteworthy that phenotypic differences were observed with SKF83959, a selective D₁-like agonist that does not stimulate adenylyl cyclase but potently stimulates PI hydrolysis (Gnanalingam et al., 1995; Panchalingam and Undie, 2001; Jin et al., 2003). This suggests little if any role for cyclic AMP activity in dopaminergic PLC stimulation.

While pharmacological agents that sufficiently discriminate among the subtypes of D₁-like receptors are still lacking, there are, nevertheless, distinguishable anatomical, biochemical, and molecular characteristics within the D₁-like receptor subfamily. For instance, the D₅ receptor is widely but discretely distributed in the brain, with a pattern that differs substantially from the distribution of D₁ receptors (Meador-Woodruff et al., 1992; Ciliax et al., 2000). Within the striatum, the D₅ receptor is expressed predominantly in cholinergic interneurons, whereas D₁ receptors predominate in GABAergic neurons (Rivera et al., 2002; Centonze et al., 2003). Unlike the D₁ receptor, D₅ receptors do not form functional complexes with A₁ adenosine receptors (Le Crom et al., 2002), and plasma membrane localization of the D₅ (but not the D₁) receptor requires N-glycosylation (Karpa et al., 1999). These observations indicate significant structural differences between the receptor subtypes, and this is consistent with the recent observation of differential physicochemical susceptibility of G_s-coupled versus G_q-coupled D₁-like sites to reducing agents or plasma membrane perturbations (Panchalingam and Undie, 2005). Thus, while there may be substantial pharmacological overlap within the D₁-like family, the two subtype members are sufficiently differentiated in structure, anatomical distribution and function

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to warrant their differential coupling to downstream signaling cascades. Nevertheless, future studies on the D₅ receptor should address whether such coupling ultimately involves receptor hetero-oligomerization, functional selectivity, or multiple G protein interactions – phenomena that could explain a receptor's ability to induce unique or multiple signaling responses.

With regard to neurobiological function, evidence suggests that the D₅ receptor is probably the D₁-like receptor subtype that mediates a range of dopamine's effects, including the regulation of peripheral blood pressure (Hollon et al., 2002), enhanced acetylcholine release in the hippocampus (Hersi et al., 2000), stimulation of pituitary prolactin secretion (Saller and Salama, 1986), and modulation of mucosal vulnerability to psychosomatic ulcerogenic insults (Hunyady et al., 2001). At the behavioral level, congenic D₅ receptor mutant mice show marked reductions in grooming, a characteristic D₁-like dopaminergic response that is nevertheless not cyclase-mediated (O'Sullivan et al., 2005). Moreover, orofacial movement topographies inducible in naïve animals by SKF83959 (which does not stimulate adenylyl cyclase), are severely disrupted in congenic D₅ mutant mice (Tomiyama et al., 2006). Hence, the D₅ receptor probably regulates a defined subset of physiological dopaminergic responses, as was previously thought for the dopamine-linked PI signaling response (Undie et al., 2000). Our present observations, therefore, should offer potential new clues toward clarifying the downstream intracellular pathways that define the neurochemical and behavioral phenotype of the D₅ dopamine receptor.

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Footnotes

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Legends for Figures

Fig. 1. Effects of D₅ receptor deletion on dopaminergic-induced inositol phosphate accumulation in brain slices. Tissue slices prepared from frontal cortex, striatum, or hippocampus of wildtype and D₅ receptor knockout (D₅KO) mice were double-labeled with [³H]inositol and [¹⁴C]cytidine and then incubated with indicated concentrations of test agonists for 60 min. Tritiated inositol phosphates were separated and quantified. Data from 3-4 separate experiments were normalized against baseline accumulations of [³H]inositol phosphate in the corresponding WT tissues and then pooled for analysis. Each bar is the mean ± SEM (N=6-8). *p<0.05; **p<0.01; ***p<0.001, *posthoc* Bonferoni comparisons of D₅KO versus WT responses at each drug concentration.

Fig 2. Effects of D₅ receptor knockout phenotype on agonist-induced CDP-diacylglycerol stimulation in brain slices. Experiments were conducted as described in Fig. 1. Diacylglycerol-derived [³H]CDP-diacylglycerol was extracted and quantified by liquid scintillation as described. Data for each tissue were collated from 3-4 separate experiments and normalized against wildtype baseline accumulations of [³H]CDP-diacylglycerol. Each bar is the mean ± SEM (N=6-8). *p<0.05; **p<0.01; ***p<0.001, *posthoc* Bonferoni comparisons of D₅KO versus WT responses at each drug concentration.

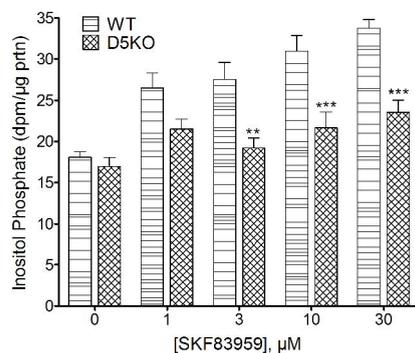
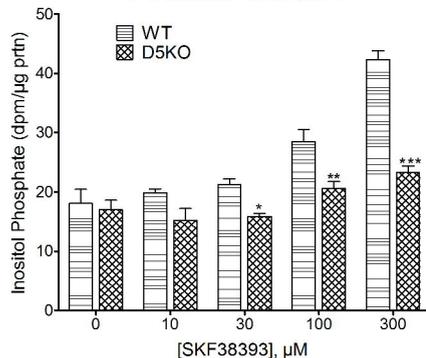
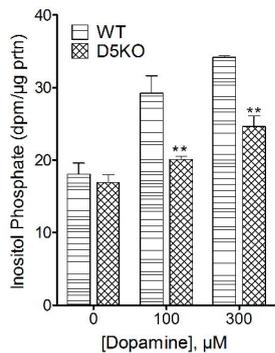
Fig. 3. Phenylephrine-stimulated inositol phosphate and CDP-diacylglycerol accumulation. Experiments were conducted as described in Fig. 1, using frontal cortex tissues from wildtype

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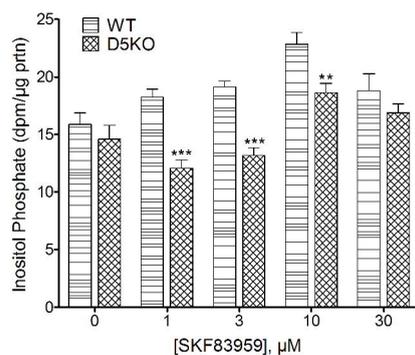
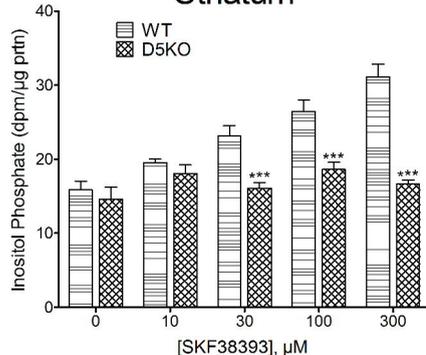
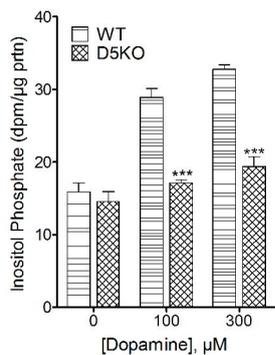
(WT) and D₅ receptor-knockout (D₅KO) mice. Each bar is the mean±SEM (N=6). ++p<0.01 compared to the respective wildtype or knockout control (0 μM phenylephrine).

Fig. 4. Effects of D₅ receptor deletion on SKF83959-induced formation of endogenous inositol-1,4,5-trisphosphate. Wildtype and D₅ receptor knockout mice were injected with the indicated dosages of SKF83959 dissolved in 5 ml/kg saline. After 20 min, the animals were killed and the brains quickly dissected and flash-frozen for subsequent assay of inositol-1,4,5-trisphosphate using the radioreceptor binding technique. Tissues from each hemisphere were processed separately and the data pooled for statistical analysis. Each bar represents the mean±SEM (N=6 hemispheres). *p<0.05; **p<0.01; ***p<0.001, *posthoc* Bonferoni comparisons of D₅KO versus WT responses at each drug concentration.

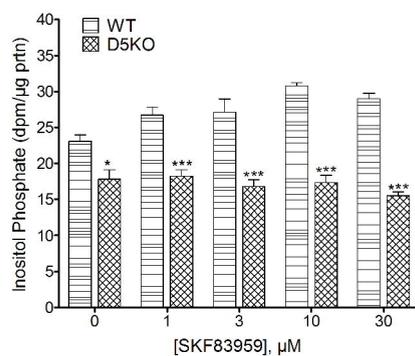
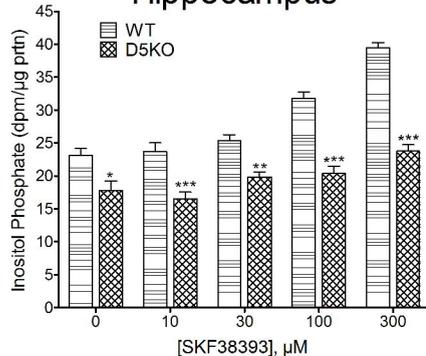
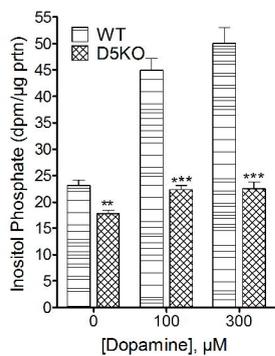
Frontal Cortex



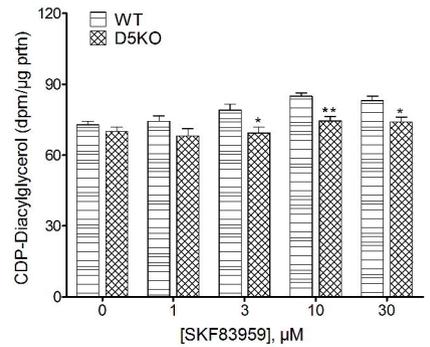
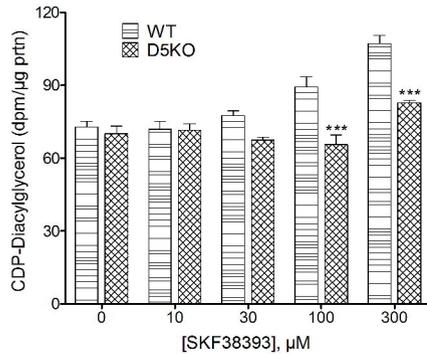
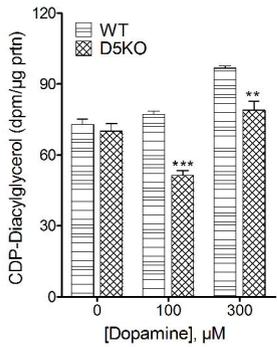
Striatum



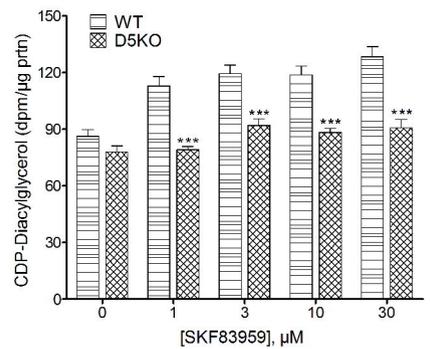
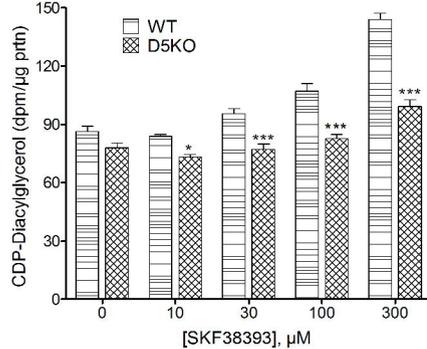
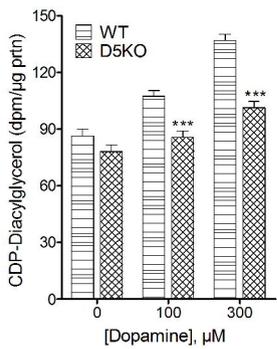
Hippocampus



Frontal Cortex



Striatum



Hippocampus

