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Functional activation by central monoamines of human dopamine D_4 receptor polymorphic variants coupled to GIRK channels in *Xenopus* oocytes

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Abstract

We studied the functional activation of different polymorphic variants of the human dopamine D_4 receptors by the three major central monoamines, dopamine, noradrenaline and serotonin. Dopamine D_4 receptors carrying two (D4.2), four (D4.4) or seven (D4.7) repeats within the third intracellular domain were co-expressed with G protein-regulated inwardly rectifying potassium channels (GIRK1) in frog oocytes. All the dopamine D_4 receptor variants coupled to oocyte $G_{i/o}$ proteins and modulated co-expressed GIRK1 channels. Monoamine-induced responses were detected as increases in voltage-clamp recorded GIRK1 currents. Dopamine, noradrenaline as well as serotonin stimulated dopamine D_4 receptors. Dose-response analysis showed that dopamine and noradrenaline are full agonists whereas serotonin acted as partial agonist. Dopamine was 5-fold more potent on D4.2 and D4.7 (EC_{50}=1 nM) than on D4.4 (EC_{50}=5 nM) suggesting that the actions of dopamine and therapeutic drugs on dopamine D_4 receptors might vary among individuals depending on their repertoire of expressed alleles. In contrast, noradrenaline and serotonin did not discriminate among dopamine D_4 receptor variants ($EC_{50\ NA}$=50 nM, $EC_{50\ 5\text{-}HT}$=1.5 μM). All monoamine effects were blocked by the specific dopaminergic D_4 antagonist (S)-(−)-4-[4-[2-(Isochroman-1-yl)ethyl]piperazin-1-yl]benzenesulfonamide (PNU101387). Sequence analyses of dopamine D_4 receptors and related monoamine receptors revealed that dopamine D_4 receptors have most aminoacidic residues necessary for binding of dopamine, noradrenaline and serotonin. Our data indicate that dopamine D_4 receptors can be pharmacologically stimulated by any the three major central monoamines.

Keywords: Dopamine D_4 receptor; Polymorphic variants; Serotonin; Noradrenaline; GIRK channels

1. Introduction

The dopamine D_4 receptor is a member of the G protein-coupled receptors superfamily (Neve, 2005; Van Tol et al., 1991) that mediates changes in neuronal excitability and synaptic plasticity in the brain (Rubinstein et al., 2001; Wang et al., 2003, 2002), and is predominantly expressed in the brain prefrontal cortex (Ariano et al., 1997; Mrzljak et al., 1996), where it is thought to play a major role in the control of integrative functions underlying the organization of complex behaviors (Fuster, 2001; Goldman-Rakic, 1995b; Rubinstein et al., 1997). Dopamine D_4 receptor activation may trigger multiple intracellular pathways including inhibition of cAMP synthesis (Seamans and Yang, 2004) and modulation of G protein-regulated ion channels (Lavine et al., 2002; Pillai et al., 1998; Werner et al., 1996).

An outstanding feature of the human dopamine D_4 receptor is its highly polymorphic nature, particularly in the third cytoplasmic domain where a variable number of 48 bp repeats and single nucleotide polymorphisms account for more than 35 different alleles already detected in the population (Grady et al., 2003). Variants carrying four tandem repeats (D4.4) account for approximately 64% of all human alleles, whereas the other two most abundant variants are D4.7 (20%) and D4.2 (8%)

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(Chang et al., 1996). Many studies have attempted to correlate polymorphic human dopamine D_4 receptor variants with psychiatric diseases and personality traits and some of them found that D4.7 variants are associated with attention deficit and hyperactivity disorder (ADHD) and with the personality trait novelty seeking (Ebstein et al., 1996; Faraone et al., 2001; Grady et al., 2003; Paterson et al., 1999). Because the third intracellular domain of G protein-coupled receptors interact with its cognate G proteins and other intracellular signaling molecules (Neve, 2005), numerous studies have also analyzed the functional and pharmacological properties of several human polymorphic dopamine D_4 receptor variants. For example, the ability of the various human dopamine D_4 receptor variants to bind dopamine, and to induce dopamine-mediated inhibition of adenylyl-cyclase activity or stimulation of GTPγS binding, was evaluated in transfected cell lines (Asghari et al., 1995; Czermak et al., 2006; Jovanovic et al., 1999). Dopamine D_4 receptor-mediated modulation of inwardly rectifying potassium channels (GIRK) was also demonstrated in heterologous expression systems (Werner et al., 1996). However, the functional coupling between different dopamine D_4 receptor polymorphic variants and GIRK has not been studied yet.

Another level of complexity in studies of dopamine D_4 receptor pharmacology and function derives from the interactions that exist between different monoamines and their receptors. Particularly, it was demonstrated that noradrenaline and adrenaline bind dopamine D_4 receptors with high affinity and induce dopamine D_4 receptor-mediated inhibition of adenylyl-cyclase (Czermak et al., 2006; Lanau et al., 1997; Newman-Tancredi et al., 1997) and that different human recombinant serotonin receptors can be activated by dopamine (Oz et al., 2003; Woodward et al., 1992).

Diverse crossed effects were also reported for dopaminergic, adrenergic or serotonergic agonists and antagonists, even for drugs showing high degrees of selectivity. In addition, it is well known that atypical antipsychotics, like clozapine, show high affinities for dopamine D2 receptors, dopamine D_4 receptors and serotonin 5-HT2 receptors (Stockmeier et al., 1993; Van Tol et al., 1991). This level of ligand-receptor promiscuity may have important pharmacological consequences, for example during treatments with psychotherapeutic drugs.

The effects of serotonin on dopamine D_4 receptors has not been investigated before, neither the actions of noradrenaline to modulate GIRK currents through dopamine D_4 receptor activation. In the present study, we used electrophysiological recording in *Xenopus laevis* oocytes, to test the ability of the three major central monoaminergic neurotransmitters to stimulate the most abundant human dopamine D_4 receptor variants and modulate GIRK currents through oocyte $G_{i/o}$ proteins.

2. Materials and methods

2.1. RNA preparation, oocyte isolation and injection

Plasmids encoding the different human dopamine D_4 receptor variants: D4.2 (αξ), D4.4 (αβθξ) and D4.7 (αβηεβεξ) (greek characters are used in order to define the kind of repeat involved in protein structure) (Jovanovic et al., 1999) and the rat GIRK1, were provided by colleagues (see acknowledgments). Full-length cDNAs, cloned in pcDNA3 (Invitrogen), were used to *in vitro* transcribe cRNA with the mMessage mMachine transcription kit (Ambion, Austin, TX, USA). cRNA solutions (0.2–0.4 μg/μl) were prepared in RNase-free H_2O and stored at −70 °C. *Xenopus laevis* (Nasco, Modesto, CA, U.S.A.) oocytes at stages V and VI were used for expression of human dopamine D_4 receptors and homomeric GIRK1 channels. Isolation and maintenance of the oocytes were carried out as previously described (Miledi, 1989). Briefly, frogs were anaesthetized with 3-aminobenzoic-acid ethylester (1 g/ml) and ovaries surgically removed. Ovaries were incubated with 200 units/ml collagenase for 50 min at room temperature (RT), and isolated oocytes were maintained in an incubator at 17 °C in Barth's medium (in mM: 88 NaCl; 0.33 $Ca(NO_3)_2$; 0.41 $CaCl_2$; 1 KCl; 0.82 $MgSO_4$; 2.4 $NaHCO_3$; 10 HEPES and 0.1mg/ml gentamycin; pH adjusted to 7.4 with NaOH). One day later, each oocyte was manually microinjected (microinjector Drummond Sci. Co., Broomall, PA, U.S.A) with 50 nl of a solution containing 10–20 ng of D4.2, D4.4, or D4.7 cRNAs plus 10–20 ng GIRK1 RNAs. Negative controls consisted of water-injected oocytes and oocytes injected with dopamine D_4 receptor cRNAs alone or GIRK1 cRNA alone. All experiments were carried out according to the Guide for the Care and Use of Laboratory Animals of the United States National Institutes of Health.

2.2. Electrophysiological recordings

Two-electrode voltage-clamp recordings were performed using an Axoclamp 2B amplifier (Axon Instruments, Union City, CA, USA). Standard glass recording electrodes were made in a Narishige PB-7 puller (Narishige Scientific Instrument Lab., Tokyo, Japan) and filled with 3 M KCl. Resistance values were approximately 1 MΩ. Current traces were acquired through an analog to digital interface (Labmaster TL-1 DMA, Scientific solutions Inc, Solon, OH, USA) and data stored on a PC using AXOTAPE software (Axon Instruments). The holding potential was set to −100 mV and ionic currents routinely recorded in oocytes injected with cRNA coding for GIRK1 in combination with one of the cRNAs coding for the different dopamine D_4 receptor variants. For recording, the cells were first placed in a 100 μl plastic chamber continuously superfused (12 ml/min) with a low potassium solution (in mM: 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, pH 7.5). Then, inward ionic currents were allowed to develop by application of a high potassium solution (in mM: 96 KCl, 2 NaCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, pH 7.5). Monoamines and other compounds used during the experiments were applied through the perfusion system at the plateau of GIRK1 currents. Monoaminergic antagonists were added 2 min before agonist's application and then simultaneously removed by washing. All the experiments were carried out at RT (24±1 °C).

2.3. Materials

Type I collagenase was purchased from Worthington (Freehold, NJ, U.S.A.). Dopamine, noradrenaline, serotonin,

L-glutamic acid, acetylcholine, L-DOPA, L-tryptophan and γ-aminobutyric acid (GABA) were from Sigma-Aldrich (St Louis, MO, U.S.A.). (*S*)-(−)-4-[4-[2-(Isochroman-1-yl)ethyl] piperazin-1-yl]benzenesulfonamide (PNU101387) was kindly provided by Pharmacia & Upjhon (Kalamazoo, MI, U.S.A). Drugs were made up freshly each day, dissolved in distilled water as 10 mM stocks and diluted in high potassium solution shortly before incubation of the oocytes. PNU101387 was diluted in acetic acid (0.01%). Up to these concentrations these solvents did not produce observed effects on the oocytes.

Test solutions: different concentrations of dopamine and serotonin chloride salts and noradrenaline bitartrate salt were added to the solutions containing HEPES. The pH of each solution was always checked and adjusted as necessary. All salts, HEPES, 3-aminobenzoic-acid ethylester, RNase-free H_2O and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.4. Analysis of the electrophysiological and pharmacological data

Data were analyzed using pClamp v.8.0 software (Axon Instruments) and statistical analyses performed using Graphpad Prism v. 3.00 (GraphPad Software, San Diego, California, U.S.A). Dose-response curves were obtained by fitting the data to the logistic equation: $I/I_{max}=A^{nH}/(A^{nH}+EC_{50}^{nH})$, where I is the peak inward current evoked by agonist at concentration A; I_{max} is the maximal response (evoked by dopamine 100 nM); EC_{50} is the agonist concentration that evoked half-maximum responses, and nH the Hill coefficient. Data were calculated as means ± standard errors of the mean (SEM). Statistical significance of EC_{50} values and nH_s were evaluated using One-Way ANOVA and Tukey's *post hoc* test. Multiple comparisons of the mean and SEM of peak current amplitudes were performed by Two-Way ANOVA.

3. Results

3.1. Stimulation of human dopamine D_4 receptors by dopamine, noradrenaline and serotonin increases GIRK currents

Ionic currents were first recorded in oocytes expressing only GIRK1 cRNA (see Materials and Methods). GIRK1 channels undergo substantial activation at −100 mV (Werner et al., 1996). Oocytes exposed to a high K^+ solution displayed currents that developed relatively fast (Fig. 1A; first trace), were sensitive to Ba^{2+} and showed a distinct voltage dependence, all characteristic features of GIRK-mediated inward K^+ currents. As shown in the same record, applications of dopamine did not induce effects on GIRK1 currents if dopamine D_4 receptor were not co-expressed as we also observed when using other monoamines and D2-like agonists (not shown). In oocytes that co-expressed D4.4 together with GIRK1, dopamine and noradrenaline, but also serotonin were capable to positively modulate GIRK1-mediated inward K^+ currents due to the

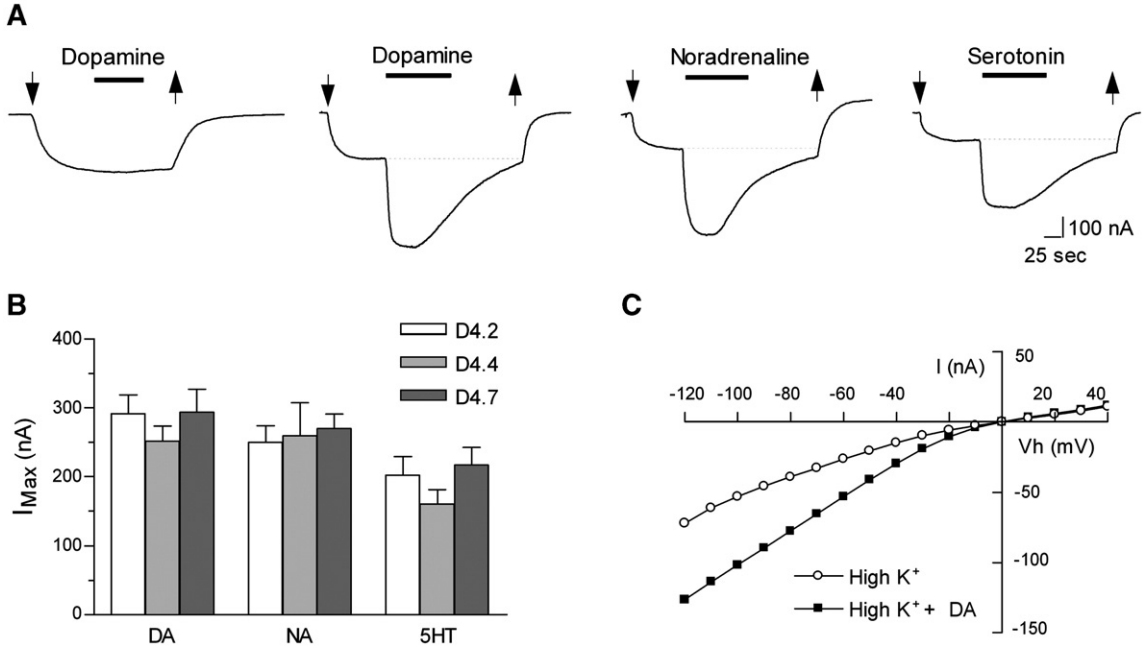

Fig. 1. *Activation of human dopamine D_4 receptors by different monoamines.* A) Representative records of the inward K^+ currents evoked by hyperpolarization in frog oocytes. The cells were steadily voltage-clamped at −100 mV and GIRK1 currents evoked by bath application of a high K^+ (96 mM) solution during the interval indicated between arrows. First trace corresponds to a current recorded from an oocyte expressing only GIRK1 channels. Dopamine (DA) failed to modulate GIRK1 in the absence of dopamine D_4 receptor (D4R). The following three records show the effects exerted by applications of dopamine (100 nM), noradrenaline (NA 10 μM) and serotonin (5-HT 100 μM) respectively, at the plateau of GIRK1 currents evoked in oocytes co-expressing D4.4 receptors and GIRK1 channels. Filled bars indicate the applications of different monoamines. (B) Maximum currents induced by dopamine (100 nM), noradrenaline (10 μM) and serotonin (100 μM) in oocytes co-expressing D4.2, D4.4 or D4.7 receptors along with GIRK1 (n=8–18). (C) Current–voltage relationship of GIRK1 currents recorded in the presence or absence of dopamine (100 nM), in oocytes co-expressing D4.4 receptors and GIRK1. The holding potential (Vh) ranged from −120 to +40 mV.

functional coupling between dopamine D_4 receptors and GIRK channels through oocyte $G_{i/o}$ proteins. The effects of 100 nM dopamine, 10 μM noradrenaline and 100 μM serotonin are shown in Fig. 1A (next three records). The three monoamines tested produced an increase in the current amplitude that persisted throughout application for approximately 25 s. These effects were rapid, relatively steady and fully reversible after agonist removal. During long and continuous exposures to monoamines (>40 s; not shown) we observed that current potentiation declined rapidly after reaching a maximum. This effect suggests some kind of response desensitization, particularly when using high concentrations of agonists. For this reason, applications of agonists were always kept as short as possible throughout the experiments. Maximum effects were observed for dopamine concentrations above 100 nM, whereas higher concentrations of noradrenaline and serotonin were needed to attain maximum effects (more than 3 and 30 μM, respectively). Despite the fact that the D4.4 variant is the most predominant allele in the human population we also investigated responses mediated by two other common alleles, D4.2 and D4.7. Dopamine evoked equivalent maximum responses on the different human dopamine D_4 receptor variants. The maximum current amplitudes evoked by 100 nM dopamine were 292.6±27.3 nA (n=12) for D4.2; 253.0±22.4 nA (n=18) for D4.4 and 295.1±33.5 nA (n=14) for D4.7 receptors. Similar results were observed for noradrenaline and serotonin (Fig. 1B). Currents evoked by 10 μM noradrenaline were 250.8±24.7 nA (n=10) for D4.2; 260.8±48.5 nA (n=10) for D4.4 and 271.4±21.0 nA (n=9) for D4.7 receptors. Meanwhile, currents evoked by 100 μM serotonin were 203.3±27.3 nA (n=9) for D4.2; 161.0±21.1 nA (n=8) for D4.4 and 218.2±25.5 nA (n=14) for D4.7 receptors. Oocytes expressing any of the dopamine D_4 receptor variants in the absence of GIRK1 were unable to evoke inward ionic currents in high K^+ and did not show any detectable response to the monoamines (not shown). Additionally, neither dopamine, noradrenaline nor serotonin, produced changes in electrical properties of water injected oocytes, such as membrane potential, membrane resistance or current baseline under voltage-clamp, even at high concentrations (not shown).

Dopamine, noradrenaline and serotonin effects on dopamine D_4 receptors were specific since the dopamine and serotonin precursors L-DOPA and L-tryptophan failed to modulate GIRK currents in similar experiments. Non-monoaminergic neurotransmitters such as L-glutamate, GABA or acetylcholine were also unable to modulate GIRK currents through dopamine D_4 receptors stimulation (data not shown).

Current–voltage (I–V) relationships were also performed. Fig. 1C illustrates a representative experiment, out of 5 performed in oocytes co-expressing D4.4 and GIRK1, in which the peaks of basal inward K^+ currents and 100 nM dopamine-stimulated currents were measured between −120 and +40 mV. GIRK1-mediated currents underwent substantial rectification and a significant activation at negative potentials, but showed negligible amplitudes at positive potentials. GIRK-mediated currents in the presence of 100 nM dopamine were larger than control currents almost all over the range of negative membrane potentials. Dopamine applications only induced changes in slopes of the I–V curves, without alteration of the reversal potentials (all measured reversal potentials were close to 0 mV) or rectification. Moreover, no significant differences were observed in the reversal potentials of the ionic currents when different dopamine D_4 receptor variants were expressed (D4.2=−0.30±0.28 mV, n=10; D4.4=0.78±0.08 mV, n=4; D4.7=0.16±0.42 mV, n=4; (N.S., one-way ANOVA test, p>0.05).

3.2. Dose-response analyses of monoamine effects on human D4.2, D4.4 and D4.7 modulating GIRK currents

The effects of dopamine, noradrenaline and serotonin on D4.2, D4.4 and D4.7 were evaluated in cumulative dose-response curves (Fig. 2A) and the corresponding EC_{50} and slope values (Hill coeficient: nH) calculated (see Materials and Methods and Table 1). Dopamine similarly activated D4.2 and D4.7 displaying nearly identical EC_{50} values (Fig. 2B and Table 1). However, D4.4 was almost five-fold less sensitive to this neurotransmitter (Fig. 2B and Table 1). In contrast, EC_{50} values for noradrenaline and serotonin showed only slight

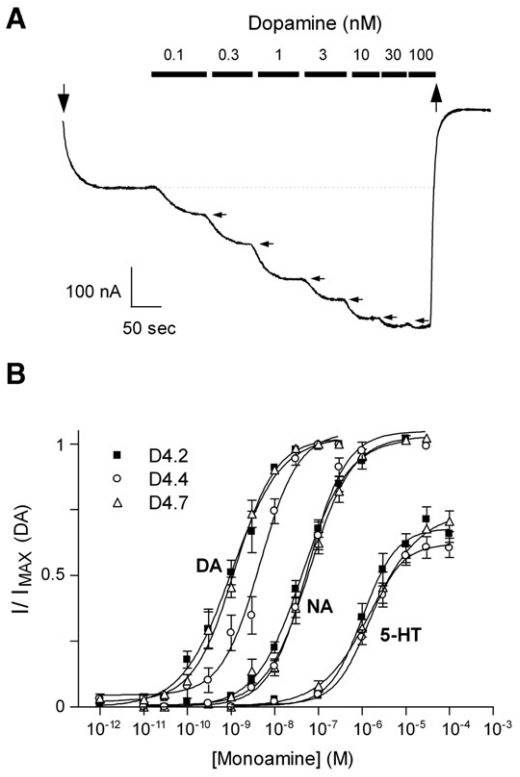

Fig. 2. *Dose-response analysis for dopamine, noradrenaline and serotonin acting on different human dopamine D_4 receptor polymorphic variants.* (A) Representative traces showing membrane current responses evoked by dopamine (DA) in oocytes expressing D4.4 receptors and GIRK1 channels. Cumulative dopamine (0.1–100 nM) applications (filled bars) increased GIRK1 current amplitude (arrows: current amplitude before the application of the next higher concentration). (B) Dose-response curves for dopamine, noradrenaline and serotonin acting on D4.2 (filled squares), D4.4 (open circles) and D4.7 (open triangles) receptors. Each data point represents the mean±SEM (n=5–17). All the experimental points were normalized to the maximum response evoked by dopamine.

Table 1
Pharmacological parameters of the actions of dopamine, noradrenaline and serotonin at human dopamine D_4 receptor polymorphic variants

	DA		NA		5-HT	
	EC_{50} (nM)	nH	EC_{50} (nM)	nH	EC_{50} (μM)	nH
D4.2	1.02±0.06	0.74±0.07	40.80±0.97	0.83±0.04	1.14±0.09	1.14±0.21
D4.4	4.89±0.28	1.11±0.17	43.50±1.76	0.91±0.07	1.42±0.05	1.07±0.11
D4.7	1.07±0.04	0.92±0.06	58.80±2.31	0.81±0.06	1.73±0.06	0.76±0.05

Data are mean±SEM values of 5 to 17 experiments. Statistical analyses: $EC_{50\ DA}$: S.D. ANOVA, $P<0.0001$. Tukey post test: D4.2 vs. D4.4, $P<0.001$; D4.2 vs. D4.7, $P>0.05$ and D4.4 vs. D4.7, $P<0.001$. $EC_{50\ NA}$: S.D. ANOVA, $P<0.0001$. Tukey post test: D4.2 vs. D4.4, $P>0.05$; D4.2 vs. D4.7, $P<0.001$ and D4.4 vs. D4.7, $P<0.001$. $EC_{50\ 5\text{-}HT}$: S.D. ANOVA, $P<0.0001$. Tukey post test: D4.2 vs. D4.4, $P<0.05$; D4.2 vs. D4.7, $P<0.001$ and D4.4 vs. D4.7, $P<0.05$.

differences that are likely biologically irrelevant (Table 1), demonstrating that only dopamine is able to differentially stimulate human polymorphic dopamine D_4 receptor variants that modulate GIRK currents. The slopes of the dose-response curves were all equivalent with *n*H close to 1 (Table 1), suggesting the presence of a single binding site for monoamines. To determine whether noradrenaline and serotonin are full or partial agonists on dopamine D_4 receptor modulation of GIRK channels we calculated the ratio between the maximum responses evoked by saturating concentrations of noradrenaline or serotonin and the maximum response elicited by dopamine at the three dopamine D_4 receptor variants (Fig. 2B). The maximum noradrenaline response/maximum dopamine response× 100 was for D4.2=102.05±1.00%; for D4.4=106.54±2.94% and for D4.7=101.45±1.66%, demonstrating that noradrenaline is a full agonist at the three human dopamine D_4 receptors. In contrast, maximum serotonin response reached approximately 65% of maximum dopamine response (D4.2=65.88±3.28%; D4.4=60.46±3.93% and D4.7=70.58±3.93%) indicating that serotonin acts as a partial agonist at all the dopamine D_4 receptor variants.

3.3. Pharmacological properties of human dopamine D4.2, D4.4 and D4.7 receptors

Dose-response curves to the specific dopamine D_4 receptors antagonist PNU101387 were performed in the presence of dopamine concentration values around the EC_{50} for each dopamine D_4 receptor variant, that is, 1 nM for D4.2 and D4.7, and 5 nM for D4.4 (Fig. 3). PNU101387 was similarly potent to block dopamine-mediated GIRK modulation by D4.2, D4.4 and D4.7, with IC_{50} values of 78.7±8.73 nM (*n*=5), 60.8±9.69 nM (*n*=9) and 47.2±5.37 nM (*n*=5), respectively (Fig. 3B). Interestingly, PNU101387 also blocked noradrenaline and serotonin actions on dopamine D_4 receptors similarly to what was observed for dopamine. Noradrenaline-evoked responses (50 nM) on dopamine D_4 receptors were inhibited 97±3% (*n*=3) in the presence of 1 μM PNU101387 whereas serotonin-evoked responses (1 μM) were inhibited 100±4% (*n*=3). These results suggest that dopamine, noradrenaline and serotonin possibly stimulate dopamine D_4 receptors by using a similar binding pocket. If this hypothesis was correct, our above demonstration that serotonin is a partial agonist at dopamine D_4 receptors could implicate serotonin as an inhibitor of dopamine-mediated dopamine D_4 receptor stimulation when saturating concentrations of dopamine are present. To test this possibility dopamine and serotonin interactions were studied in two different scenarios, first at concentrations near their respective EC_{50} (Fig. 4A) and then at saturating conditions (Fig. 4B). Fig. 4A shows a representative experiment, out of 3, where the application of 1 μM serotonin on-top of a response previously elicited by 1 nM dopamine increased the current amplitude demonstrating an additive effect. Fig. 4B illustrates a similar experiment in which both serotonin and dopamine were applied at concentrations that, individually, produce maximum effects (100 μM serotonin and 100 nM dopamine). In this case, on-top application of serotonin decreased the current amplitude induced by dopamine. These results are in agreement with

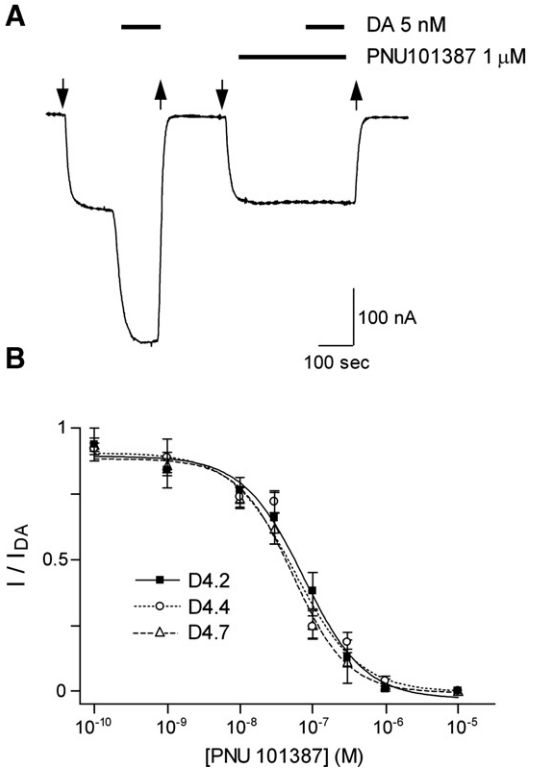

Fig. 3. *Inhibition of dopamine D_4 receptor-mediated dopamine effects on GIRK currents by the specific antagonist PNU101387.* (A) Representative trace showing the potentiation observed in GIRK1 current amplitude after an on-top application (indicated with a bar) of dopamine (DA 5 nM) in an oocyte expressing D4.4 receptors and GIRK1, before and after pre-incubation (2 min) with PNU101387 (1 μM). This antagonist prevents D4.4 receptor-mediated modulation of GIRK1 channel gating. (B) Curves of inhibition for PNU101387 acting at the D4.2 (filled squares), D4.4 (open circles) or D4.7 (open triangles) receptors. Each data point represents the mean±SEM (*n*=2–9). Currents were normalized to the maximum response evoked by dopamine.

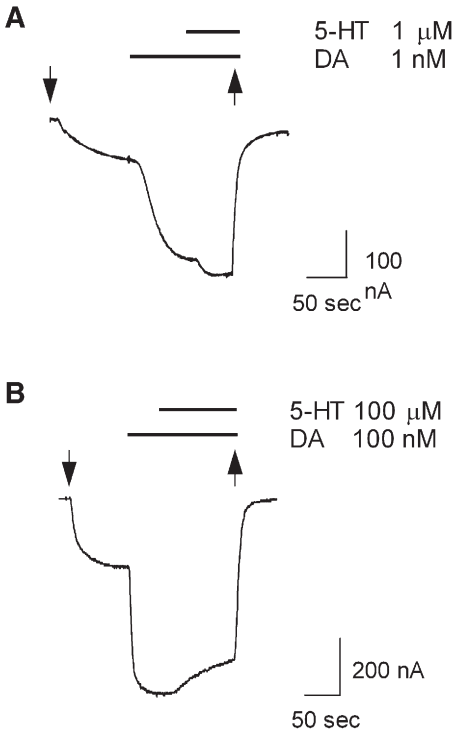

Fig. 4. *Serotonin acts as a partial agonist of the human dopamine D_4 receptor.* Representative recordings showing the effects on GIRK1 currents after co-application of different concentrations of serotonin (5-HT) and dopamine (DA) (indicated with bars) in oocytes expressing D4.4 receptors and GIRK1. (A) Serotonin increases dopamine responses at monoamine concentrations around EC_{50}. (B) Serotonin inhibits dopamine responses at monoamine saturating concentrations.

data obtained from dose-response curves for serotonin (Fig. 2B) and confirmed the partial agonist nature of serotonin on dopamine D_4 receptors. Thus, serotonin may induce an additive or inhibiting effect on dopamine-induced dopamine D_4 receptor-mediated modulation of GIRK currents depending on the relative concentrations of both monoamines.

4. Discussion

In this study we carried out a functional analysis of the effects of the three major central monoamines on human dopamine D_4 receptor polymorphic variants expressed in *Xenopus laevis* oocytes. The principal findings of this study are: 1) the three most abundant human dopamine D_4 receptor polymorphic variants coupled to and modulated co-expressed GIRK1 channels. 2) Serotonin, in addition to dopamine and noradrenaline, was able to positively modulate GIRK channels gating through dopamine D4.2, D4.4 and D4.7 receptors stimulation. However, serotonin was less potent on all dopamine D_4 receptor variants than dopamine and noradrenaline. 3) Serotonin acted as a partial agonist, whereas dopamine and noradrenaline behaved as full agonists, at all the dopamine D_4 receptor variants. 4) Only dopamine displayed different affinities for the various dopamine D_4 receptor forms, being approximately 5-fold more potent on D4.2 and D4.7 than on D4.4 to modulate co-expressed GIRK1 channels. 5) The selective dopamine D_4 receptor antagonist PNU101387 completely prevented the effects of dopamine, noradrenaline and serotonin, indicating that the three monoamines probably stimulated dopamine D_4 receptors using the same binding pocket. 6) Experiments using on-top applications of serotonin on dopamine-elicited responses demonstrated that serotonin may enhance or inhibit dopamine stimulation of dopamine D_4 receptors depending on the relative concentration of both neurotransmitters. These results are consistent with the partial agonist profile proposed for serotonin.

4.1. Functional significance of human dopamine D_4 receptor polymorphic complexity

The human dopamine D_4 receptor gene shows an extraordinary variability in its coding sequence, with multiple polymorphic alleles present in the population. A model accounting for the evolution of the human dopamine D_4 receptor gene and the biochemical and physiological impact that dopamine D_4 receptor variants may exert was recently proposed (Wang et al., 2004). However, the role that this variation might play in signaling neurotransmitter actions and ultimately determining behavioral properties is not known.

We demonstrated here that the three most abundant human dopamine D_4 receptor polymorphic variants efficiently coupled to and modulated GIRK1 channels. However, the importance of dopamine D_4 receptor-mediated GIRK modulation for control of neuronal excitability will require to be investigated by using other experimental approaches.

We also showed here that dopamine was approximately 5-fold more potent on human D4.2 and D4.7 than on D4.4 to stimulate GIRK1 currents. Functional differences among the human dopamine D_4 receptor variants were previously reported through *in vitro* assays such as dopamine-mediated coupling to adenylyl-cyclase, clozapine binding and stimulation of the GTPγS binding (Asghari et al., 1995; Czermak et al., 2006; Jovanovic et al., 1999; Van Tol et al., 1992). The present results suggest that individuals expressing the D4.2 or D4.7 alleles may have an increased sensitivity to dopamine respect to those expressing D4.4 variants. The involvement of the third cytoplasmic loop of dopamine D_4 receptors in protein–protein interactions such as receptor oligomerization, heteromerization or associations with other membrane or cytoskeletal proteins might be critical (Lavine et al., 2002; Lee et al., 2000; Neve, 2005; Oldenhof et al., 1998). Therefore, different dopamine potencies among polymorphic dopamine D_4 receptors are significant because they may contribute to inter-individual variation in factors controlling normal dopaminergic transmission and possibly the response to therapeutic agents.

4.2. Dopamine, noradrenaline and serotonin actions on human dopamine D_4 receptors

We showed here that three different human dopamine D_4 receptor polymorphic variants could be activated by any of the three major brain monoamines to modulate GIRK1 channels. A comparison of the relative potencies displayed by dopamine, noradrenaline and serotonin (Fig. 2) indicates a primary role for

dopamine in dopamine D_4 receptor stimulation, but a substantial activation of the dopamine D_4 receptors can be obtained with noradrenaline and serotonin at low micromolar concentrations. The antagonism of noradrenaline and serotonin actions by PNU101387 suggests that these monoamines are probably acting at a similar binding pocket. Three-dimension structural models and mutational studies of human dopamine D2, serotonin 5HT2B and adrenergic ADRB2 receptors predicted that interacting residues located in transmembrane helices 3, 4, 5, and 6 are critical in determining the ligands binding site (Freddolino et al., 2004; Kalani et al., 2004; Manivet et al., 2002). To further understand the relatively poor selectivity of dopamine D_4 receptors for central monoamines, in contrast to the high selectivity showed by dopamine D2 receptors (Van Tol et al., 1991), we investigated whether it carried the essential aminoacidic residues needed for dopamine, noradrenaline and serotonin shown in those studies (Freddolino et al., 2004; Kalani et al., 2004; Manivet et al., 2002). To this end, we

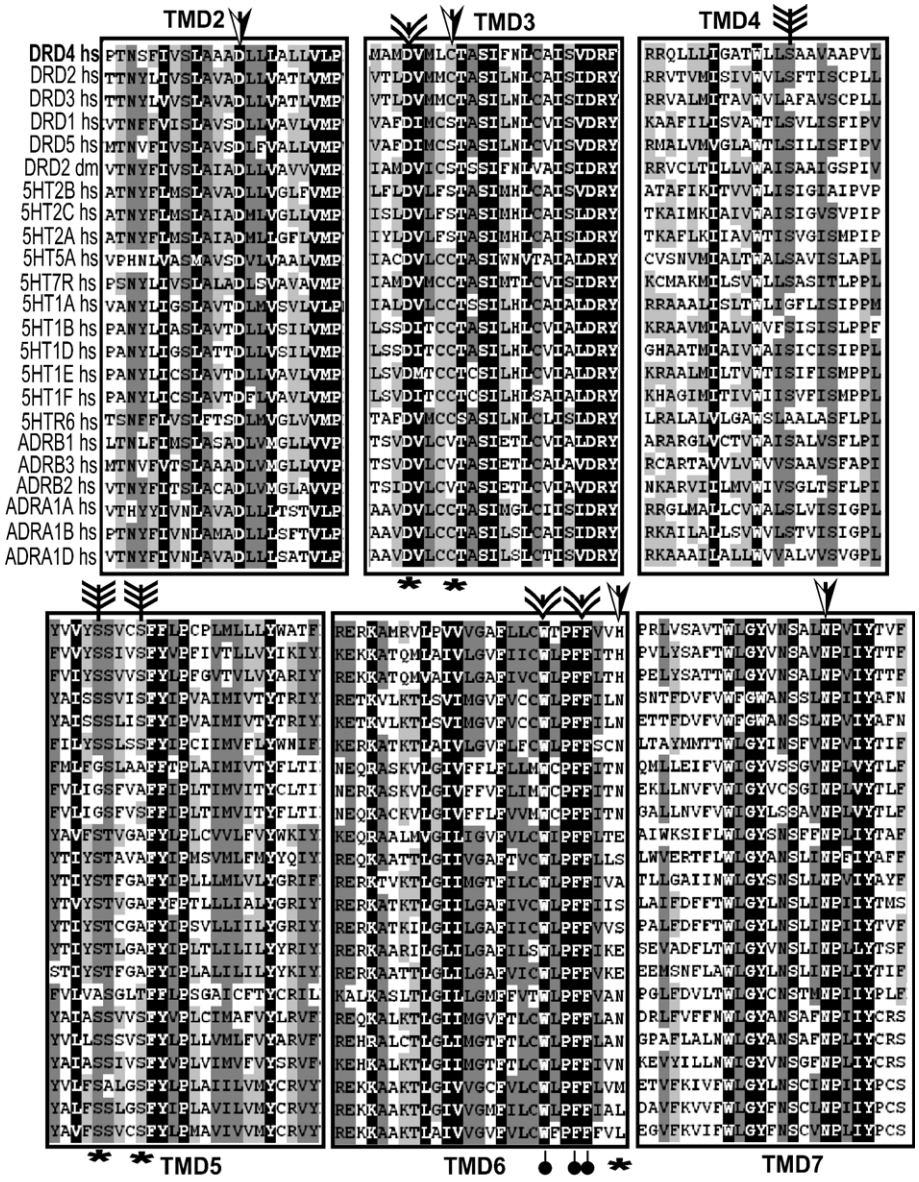

Fig. 5. *Sequence analysis of dopamine D_4 receptor and its more closely related G protein-coupled receptors*. Amino acid sequence alignment of TMDs 2, 3, 4, 5, 6 and 7 showing key amino acid sites for the binding of dopamine and noradrenaline (triple line arrows), serotonin (black and white arrows). Sites indicated by double arrows are involved in the binding of the three ligands. Black dots indicate residues involved in direct interactions with the ligands. Stars show residues forming the hydrophobic pocket for monoamine binding. Note that the two residues not conserved between dopamine D_4 receptors and serotonin 5-HT2B receptors [S139 (TMD3) and N344 (TMD6) of the 5HT2B human sequence showed low conservation among serotonin receptors. S139 is replaced for cysteine in the majority of serotonin receptors, whereas this residue is either, cysteine or serine in all dopamine receptors. Abbreviations: DRD4, DRD2, DRD3, DRD1, DRD5: dopamine receptor 4, 2, 3, 1 and 5 respectively; 5HT2A, 5HT2B, 5HT2C, 5HT5A, 5HT7R, 5HT1A, 5HT1B, 5HT1D, 5HT1E, 5HT1F: serotonin receptors; ADRB1, ADRB2, ADRB3: adrenergic receptor β1, β2 and β3 respectively; ADRA1A, ADRA1B, ADRA1D: adrenergic receptor α1A, α1B and α1D respectively; hs: *Homo sapiens*; mm: *Mus musculus*; gg: *Gallus gallus*; dm: *Drosophila melanogaster*. beta 1, and 3 respectively; ADRA: adrenergic receptor alpha; hs: *Homo sapiens*; mm: *Mus musculus*; gg: *Gallus gallus*; dm: *Drosophila melanogaster*.

performed a sequence alignment of the transmembrane domains (TMDs) of several monoamine receptors and observed that the dopamine D_4 receptor has the essential residues involved in dopamine binding, as well as all the key sites that participate in the binding of noradrenaline (Fig. 5). This finding is in agreement with our functional data showing that both neurotransmitters are full agonists at the dopamine D_4 receptor. In addition, Fig. 5 shows that the dopamine D_4 receptor has 5 out of 7 residues that have been determined to be critical for serotonin binding (Manivet et al., 2002). We observed the highest level of conservation among residues forming the hydrophobic pocket which are necessary for the binding of all monoamines (black dots). On the other hand, residues that have been predicted to directly interact with the ligands (black stars) show different degrees of conservation and most likely determine the affinity for each monoamine. Interestingly, residues S196 and S200 of dopamine D_4 receptor (Fig. 5, TMD5 black stars and triple line arrows) are conserved among all dopamine and adrenergic receptors but not in serotonin 5-HT2 receptors, indicating that they could determine the higher affinity that catecholaminergic receptors have for dopamine and noradrenaline. Further mutational-functional analysis of dopamine D_4 receptors will help to address these questions.

The relative modest selectivity observed between monoaminergic neurotransmitters and receptors has also raised the hypothesis that central monoamines may act as ligands on non-cognate receptors (Cornil et al., 2005; Czermak et al., 2006; Lanau et al., 1997; Newman-Tancredi et al., 1997; Oz et al., 2003), but most of these studies the same as our experiments were carried out using *in vitro* models. In fact, many reports have suggested the existence of a variety of interactions between monoaminergic pathways. For instance, the uptake of dopamine by the noradrenaline transporter followed by co-release of noradrenaline and dopamine was demonstrated in noradrenergic terminals of the prefrontal cortex (Carboni and Silvagni, 2004; Devoto et al., 2001; Yamamoto and Novotney, 1998). Similarly it has been shown that dopamine transporters may uptake serotonin when the extracellular concentration of this transmitter is sufficiently elevated and then, striatal dopaminergic terminals co-release dopamine and serotonin (Zhou et al., 2005). In view of our results it is also important to consider that alternatively an excess of serotonin may occur after the administration of drugs used in the treatment of anxiety and depression (i.e: fluoxetine) and after the consumption of drugs of abuse (Carboni and Silvagni, 2004; White et al., 1996; Zhou et al., 2005). Given the fact that the dopamine D_4 receptor is expressed in a region of the prefrontal cortex that receives convergent innervation of dopamine, noradrenaline and serotonin neurons (Ariano et al., 1997; Emson and Koob, 1978; Goldman-Rakic, 1995a; Mrzljak et al., 1996; Reader, 1981), the ability of the three central monoamines to interact with dopamine D_4 receptors may potentially have important physiological and/or pharmacological consequences (Benes et al., 2000; Seutin, 2005). However, additional *in vivo* experiments will be necessary to address all these issues.

In conclusion, we provide here further evidence for the interaction of the three major monoamines at the human dopamine D_4 receptors. The significance of these interactions remains to be further investigated using different experimental models, as well as their implications for the mechanism of action of antipsychotics, antidepressants and drugs of abuse.

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