RESEARCH PAPER

Trace amines depress D₂-autoreceptor-mediated responses on midbrain dopaminergic cells

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Background and purpose: Although trace amines (TAs) are historically considered 'false neurotransmitters' on the basis of their ability to induce catecholamine release, there is evidence that they directly affect neuronal activity via TA receptors, ligand-gated receptor channels and/or σ receptors. Here, we have investigated the effects of two TAs, tyramine (TYR) and β-phenylethylamine (β-PEA), on electrophysiological responses of substantia nigra pars compacta (SNpc) dopaminergic cells to the D₂ receptor agonist, quinpirole.
Experimental approach: Electrophysiological recordings of D₂ receptor-activated G-protein-gated inward rectifier K⁺ channel (GIRK) currents were performed on dopaminergic cells from midbrain slices of mice and on Xenopus oocytes expressing D₂ receptors and GIRK channels.
Key results: TYR and β-PEA reversibly reduced D₂ receptor-activated GIRK currents in a concentration-dependent manner on SNpc neurones. The inhibitory effect of TAs was still present in transgenic mice with genetically deleted TA₁ receptors and they could not be reproduced by the selective TA₁ agonist, o-phenyl-3-iodotyramine (O-PIT). Pretreatment with antagonists of σ1 and σ2 receptors did not block TA-induced effects. In GTPγS-loaded neurones, the irreversibly-activated GIRK-current was still reversibly reduced by β-PEA. Moreover, β-PEA did not affect basal or dopamine-evoked GIRK-currents in Xenopus oocytes.
Conclusions and implications: TAs reduced dopamine-induced responses on SNpc neurones by acting at sites different from TA₁, σ-receptors, D₂ receptors or GIRK channels. Although their precise mechanism of action remains to be identified, TAs, by antagonizing the inhibitory effects of dopamine, may render dopaminergic neurones less sensitive to autoreceptor feedback inhibition and hence enhance their sensitivity to stimulation.
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Introduction

The trace amines (TAs), tyramine, β-phenylethylamine (β-PEA), octopamine and tryptamine, are a class of endogenous compounds similar to classical monoamine neurotransmitters in their structure, metabolism and tissue distribution (Berry, 2004). Several lines of evidence suggest a link between TAs and the mesencephalic dopaminergic system. Thus, a relatively high level of tyramine (TYR) and β-PEA is present in nigrostriatal and mesolimbic regions of the brain (Boulton, 1976; Paterson *et al.*, 1990). Moreover, β-PEA is synthesized by dopaminergic neuron (Greenshaw *et al.*, 1986) and specific high affinity binding sites for β-PEA (Richard *et al.*, 1982) and TYR (Vaccari, 1986) have been described in the striatum and other dopamine-rich areas. These observations are consistent with the notion that TAs might play an important role in the control of movement, mood, reward and cognition (Boulton, 1980; Davis and

Boulton, 1994; Bergman *et al*., 2001; Sotnikova *et al*., 2008). Furthermore, a dysregulation in the actions of TAs has been linked to various neuropsychiatric disorders including schizophrenia, attention-deficit/hyperactivity disorders, depression and Parkinson's disease, all of which are characterized by altered activity of monoaminergic networks (Boulton, 1980; Davis and Boulton, 1994; Branchek and Blackburn, 2003; Burchett and Hicks, 2006; Millan, 2006)

Although TAs are phylogenetically older than classical biogenic amines, as mentioned earlier, they are structurally related and share metabolic pathways (Paterson *et al*., 1990). In fact, TAs are primarily synthesized by the enzyme L-amino acid decarboxylase (AADC) and levels in the brain depend on the activity of monoamine oxidase (MAO). Interestingly, TAs are stored in nerve terminals with classical biogenic amines and can be co-released in a vesicular and non-vesicular manner (Kosa *et al*., 2000). In spite of these intriguing observations, the physiological roles and mechanisms of action of TAs in the mammalian brain remain poorly understood (Borowsky *et al*., 2001; Bunzow *et al*., 2001; Premont *et al*., 2001; Branchek and Blackburn, 2003; Burchett and Hicks, 2006; Millan, 2006).

In fact, the best-characterized effect of TAs consists of their ability to release, in an amphetamine-like manner, catecholamines via their displacement from synaptic vesicles and reversal of plasma membrane transporters. Furthermore, of particular pertinence to dopaminergic transmission, it has been demonstrated that TAs are able to release dopamine from synaptosomes (Raiteri *et al*., 1977) and striatal brain slices (Dyck, 1983). Moreover, *in vivo* studies (Philips and Robson, 1983; Bailey *et al*., 1987) have shown that β-PEA releases dopamine in the striatum (Philips and Robson, 1983) and nucleus accumbens (Nakamura *et al*., 1998) and induces ipsilateral rotation in unilateral 6-hydroxydopamine-lesioned animals (Barroso and Rodriguez, 1996). Consistent with these findings, we have observed inhibitory effects of TYR and β-PEA on dopaminergic perikarya that appear to be mediated by the extracellular release of dopamine (Geracitano *et al*., 2004). Moreover, indirect effects of TYR, mediated by the release of dopamine acting at D_2-like receptors, depolarize subthalamic neurones and depress GABAergic transmission (Zhu *et al*., 2007).

An additional interesting dimension to the physiology of TAs is the possibility that they may exert direct actions *independently* from their neurotransmitter releasing properties. Their modulation of neuronal activity could be related to the activation of G protein-coupled receptors (GPCRs) and/or ligand-gated ion channels. Indeed a novel family of TA receptors was recently identified of which two, TA_1 and TA_2 (Maguire *et al*., 2009), were found to be sensitive to TAs (Borowsky *et al*., 2001; Bunzow *et al*., 2001). TA_1 are positively coupled to Gs protein, leading to the intracellular accumulation of cAMP upon activation of adenylyl cyclase. Interestingly, recent experimental evidence suggests that TA_1 (also called 'TA associated receptor 1', TAAR1) modulate monoaminergic transmission (Sotnikova *et al*., 2008; Lindemann *et al*., 2008; Xie and Miller, 2009) by an effect on monoamine transporters (Xie *et al*., 2007, 2008; Xie and Miller, 2009) and the firing activity of mesencephalic dopaminergic neurones (Lindemann *et al*., 2008).

Apart from TA_1 receptors coupled to G-proteins, it is conceivable that ionotropic receptors are activated by TAs as, in invertebrates, ligand-gated chloride channels are opened by TAs (Pirri *et al*., 2009; Ringstad *et al*., 2009). Moreover, it has been demonstrated that both β-PEA and TYR activate σ receptors (Fontanilla *et al*., 2009). σ-Receptors were initially described as a subtype of opioid receptor (Martin *et al*., 1976) but are now recognized to be a distinct family comprising two subtypes (σ1 and σ2) possessing distinct biochemical and pharmacological profiles (Itzhak *et al*., 1991; Monnet and Maurice, 2006). Therefore, TAs may fine-tune neuronal activity by directly acting on σ receptors. In fact, the activation of σ1 and σ2 receptors has been reported to inhibit various types of K^+ and Ca^{2+} ion channels (Nguyen *et al*., 1998; Zhang and Cuevas, 2002) through direct protein-protein interactions in a G-protein-independent manner unrelated to protein kinases (Lupardus *et al*., 2000; Zhang and Cuevas, 2005).

From the comments mentioned earlier, it appears that TAs may exert a direct influence upon neuronal activity. In the present manuscript, we present evidence that both TYR and β-PEA reduce dopamine-induced responses in midbrain dopaminergic neurones via a G protein-independent signalling mechanisms mediated neither by TA_1 nor by σ receptors.

Methods

Slice preparations
The electrophysiological recordings were made from dopaminergic neuron of SNpc in acute slices of ventral midbrain obtained using standard procedures (Mercuri *et al*., 1995). C57BL6 mice and TA_1 KO mice (background C57BL6; 18–25 days old) were anaesthetized with halothane and decapitated. All experiments were carried out in accordance with the international guidelines on the ethical use of animals from the European Communities Council Directive of 24 November 1986 (86/609/EEC) and Comitato Etico of the Tor Vergata University, Rome. The brain were rapidly removed from the skull and a tissue block containing the midbrain was mounted on an agar block and immersed in cold artificial cerebrospinal fluid (aCSF) at 8–10 C°. The aCSF contained 126 mM NaCl, 2.5 mM KCl, 1.2 mM $MgCl_2$, 2.4 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 24 mM $NaHCO_3$ and 10 mM glucose and was saturated with 95% O_2–5% CO_2 (pH 7.4). Horizontal slices (250 μm) of the ventral midbrain, containing the substantia nigra, were cut using a vibratome (Leica VT1000S, Leica Microsystems, Wetzlar, Germany). Slices were maintained in aCSF at 33.0 ± 0.5 C° for 45 min before being transferred to the recording chamber.

Electrophysiology
Intracellular recordings from midbrain SNpc dopaminergic neurones were made with sharp microelectrodes. The experiments were performed at 33.0 ± 0.5 C° in a recording chamber, containing aCSF flowing at a rate of 2.5–3 mL·min^{-1} and continuously oxygenated, on the stage of an upright microscope (Axioscope FS, Zeiss, Gottingen, Germany), equipped for infrared video microscopy (Hamamatsu, Tokyo, Japan) in order to allow the direct visualization of the recorded neuron. Neu-

rones, selected for their location and morphology, were identified as dopaminergic by their electrophysiological properties, such as the presence of a regular spontaneous firing activity (0.5–4 Hz), a large inward current (Ih) in response to hyperpolarizing voltages and a membrane hyperpolarization/outward current caused by the application of dopamine (10–30 μM) or quinpirole (10–300 nM) (Mercuri *et al*., 1995). The electrodes used were filled with 2 M KCl and had a tip resistance of 30–80 MΩ. Recordings were made with an Axoclamp 2B amplifier (Axon instruments, Foster City, CA, USA) using Clampex software and a Digidata 1200B connected to a PC.

Voltage clamp experiments were performed while maintaining the membrane voltage between –60 and –65 mV by hyperpolarizing current injection in order to avoid spontaneous spike triggering. The single-electrode voltage-clamp technique (Lacey *et al*., 1987) was used in preference to the whole-cell patch-clamp technique because the D_2 receptor-mediated outward current could be stably followed for more then 1 h with little fading with the former method. In some experiments, current–voltage (I/V) relationships were constructed by applying voltage pulses of 14–16 mV increments from about –45 to –130 mV in voltage-clamped neurones in control conditions, during the application of quinpirole and the co-application of quinpirole plus β-PEA.

For the experiments performed to verify the potential involvement of G protein, the dopaminergic neurones were loaded with GTPγS (1 mM) dissolved in the microelectrode pipette filling solution. To inhibit dopa decarboxylase and MAO B, midbrain slices were incubated in aCSF (at 33.0 ± 0.5 C°) containing carbidopa (300 μM) and selegiline (30–100 nM), respectively, for more than an hour to ensure complete enzymatic inhibition and then transferred to the recording chamber.

Oocytes preparation and RNA injection

Xenopus laevis care and handling were in accordance with the highest standards of institutional guidelines in compliance with both national and international laws and policies. Frogs underwent no more than two surgical procedures, separated by at least 3 weeks. Frogs were anaesthetized with an aerated solution of 3-aminobenzoic acid ethyl ester. Stages V–VI oocytes were isolated and stored at 16°C in fresh ND96 medium (NaCl 96 mmol·L^{-1}, KCl 2 mmol·L^{-1}, MgCl$_2$ 1 mmol·L^{-1}, CaCl$_2$ 1.8 mmol·L^{-1}, HEPES 5 mmol·L^{-1}, gentamicin 50 μg·mL^{-1}). D_2 receptors and Kir 3.2 channel subunits composing GIRK2 channels the same as those expressed in SNpc neurones of adult mice were subcloned into the oocyte expression vector pBF, which provides 5' and 3' untranslated regions from the *Xenopus* β-globin gene flanking a polylinker containing multiple restriction sites. Capped mRNAs were synthesized *in vitro* by using the SP6 mMESSAGE mMACHINE kit (Ambion). Kir3.2 mRNA (50 nL) and D_2 receptor mRNA (50 nL) were injected into the oocytes (Nanoject, Drummond, Broomall, PA, USA). All the reagents were supplied by Sigma (Milan, Italy).

Electrophysiology

Electrophysiological recordings and data analysis were performed as previously described (Pessia *et al*., 1996). Briefly,

two-electrode voltage-clamp recordings (TEVC) were obtained from *Xenopus* oocytes at ~22°C, 2–8 days after injection. A GeneClamp 500 amplifier (Axon Instruments) interfaced to a PC computer with an ITC-16 interface (Instrutech Corp., Port Washington, NY USA) was used. Microelectrodes were filled with KCl 3 M and had resistances of 0.1–0.5 MΩ. Standard recording solution contained 90 mM KCl, 3 mM MgCl$_2$, 10 mM Hepes (pH 7.4) was used, unless otherwise stated. Currents were evoked by voltage commands from a holding potential of –10 mV, delivered in –10 mV increments from +50 to –120 mV, unless otherwise stated. The recordings were filtered at 2 kHz and acquired at 5 kHz with Pulse software (HEKA elektronik GmbH, Lambrecht, Germany). Data analysis was performed by using IGOR (Wavemetrics, Lake Oswego, OR, USA), PulseFit (HEKA elektronik GmbH) and Kaleida-Graph (Synergy Software, Reading, PA, USA). Leak and capacitative currents were subtracted using a P/4 protocol.

Mice genetically lacking TA_1 receptors

Mice genetically deleted of TA_1 receptors were custom-generated by GenOway (Lyon, France) employing a conventional beta-lac strategy, as employed by others (Lindemann *et al*., 2008). Briefly, the TA_1 receptor coding sequence was replaced by a cDNA encoding IRES-LacZ-NeoTk cassette. 129SvPas ES cells were transfected and chimeras obtained from C57BL/6 blastocysts. Homozygous mice showing a pure C57BL/6J genetic background were bred and back-crossed for 8 generations prior to utilization of congenic knockout and wild-type mice (De Groote *et al*., 2007; Millan *et al*., 2008). Adult knockout mice were healthy and normal with no apparent phenotype, hence indistinguishable from their wild-type counterparts. The effectiveness of gene deletion was validated by employing standard histoenzymological detection of b-galactosidase on 30 μm saggital brain sections of knockout mice, as well as real-time polymerase chain reaction (PCR) quantification of TA_1 receptor transcripts in defined (micro-punched) cerebral regions, including the SNpc (Millan *et al*., 2008).

Drugs

All drugs were prepared in stock solutions and bath-applied at known concentrations via a three-way tap system. A complete exchange of the solution in the recording chamber occurred in about 1 min. In some experiments GTPγS (1 mM) was infused into the cytosol through the recording pipettes. The following substances were used: dopamine hydrochloride, β-phenylethylamine (β-PEA), tyramine (TYR), d-amphetamine sulphate (AMPH), cocaine hydrochloride, S-(–)-carbidopa, R-(–)-selegiline hydrochloride, GTPγS tetralithium salt were purchased from Sigma. (–)-Quinpirole hydrochloride, SM-21 maleate, BD1047 dihydrobromide were obtained from Tocris Cookson Inc. (Bristol, UK). o-Phenyl-3-iodotyramine (*O*-PIT) was synthesized by Servier chemists.

Data analysis

Numerical data are expressed as mean ± SEM. Student's *t*-test for paired and unpaired observations was used to compare

data. The threshold level of significance for all analyses was $P < 0.05$. To estimate the IC_{50} and maximal response, concentration-response curves were fitted using the logistic equation $Y = a/\{1 + \exp[-(x - x0)/b]\}$ where a is maximal effect, b is minimum effect, x is drug concentration, $x0$ is the concentration attaining half-maximal effect.

Nomenclature
The nomenclature of all molecular targets (receptors, ion channels, enzymes, etc.) cited in this work conforms to the *British Journal of Pharmacology*'s Guide to Receptors and Channels (Alexander *et al*., 2009).

Results

TAs reduce D_2-activated responses on dopaminergic neurones
Intracellular recordings with sharp microelectrodes were made from dopaminergic neurones in the mice SNpc ($n = 92$). The electrophysiological and pharmacological characteristics of these cells have been described previously (Lacey *et al*., 1989; Mercuri *et al*., 1995).

Bath application of dopamine (10–30 µM) and the selective D_2 receptor agonist, quinpirole (0.01–1 µM), activated the somatodendritic receptors, which led to a membrane hyperpolarization/outward current because of opening of $G_{i/o}$–gated K^+ channels (Lacey *et al*., 1987). In the present study, we routinely perfused quinpirole (300 nM) to induce a maximal and constant GIRK activation in single-electrode voltage-clamped neurones held at –60 mV, and evaluated whether the co-application of TAs modulated the quinpirole-induced outward current. Both β-PEA and TYR reversibly reduced the amplitude of the sustained outward current (78.9 ± 3.6 pA) caused by the continuous perfusion of quinpirole (300 nM) ($n = 59$). In particular, co-application (4–7 min) of β-PEA (10–300 µM) or TYR (30–300 µM) inhibited the D_2 receptor-activated GIRK current (Figure 1A, D). The effect of the TAs peaked at 3–5 min and washed out in 8–15 min. It was reproducible and concentration-dependent (Figure 1A, B, E). The IC_{50} value for β-PEA was 82.9 ± 9.6 µM and the maximal inhibition produced by this drug was obtained at a concentration of 300 µM (84.2 ± 8.3%, $n = 8$). Interestingly, an inhibitory effect similar to that of TAs was produced by the dopamine releaser AMPH (30 µM; Figure 1C, F) but not by the dopamine reuptake blocker cocaine (10 µM; Figure 1D). In particular AMPH perfusion (30 µM, $n = 7$) reduced the D_2 receptor-activated GIRK currents by 41.2 ± 2.1%, whereas cocaine (10 µM, $n = 4$) was unable to inhibit quinpirole-induced currents and to prevent the depressant effects produced by TAs (Figure 1D, F).

In order to verify whether a reduction of TA catabolism could influence their inhibitory effect on the quinpirole-activated GIRK current, we performed recordings in midbrain slices preincubated with the MAO B inhibitor, selegiline. Indeed, in slices preincubated with selegiline (30–100 nM for more then 1 h) the inhibitory effect on GIRK currents was achieved at lower concentrations of the TAs. The concentration-response curve for β-PEA was shifted to the left (Figure 2A) and the IC_{50} value reduced to 19.7 ± 8.5 µM

($n = 7$) indicating that inhibition of MAOs reduces the effective doses of the TAs.

In another set of experiments, the irreversible AADC inhibitor carbidopa was used to reduce the synthesis of both dopamine and TAs. Carbidopa, which abolishes the indirect dopamine-mediated effects of TAs on dopaminergic neurones (Geracitano *et al*., 2004), by reducing the rate of synthesis of TAs, might unmask a greater effect of exogenously applied TAs. In fact, under these conditions the TAs still inhibited the D_2 receptor-activated GIRK current and the percentage reduction was slightly higher compared with control conditions (Figure 2B). In particular β-PEA (100 µM) produced an inhibition of 73.3 ± 10.4% ($n = 7$) in carbidopa-treated cells versus 49.5 ± 5.1% ($n = 18$; $P < 0.05$) in control cells.

The effects of TYR and β-PEA are not caused by the activation of TA_1 receptors
It was possible that the effects of TAs on D_2 receptor-induced outward current were the result of the activation of TA_1 receptors. We addressed this issue by utilizing mice genetically knocked-out for TA_1, as verified both by PCR quantification of cerebral levels of TA_1 (complexly absent in the SNpc and other CNS regions of knockout vs. wild-type mice) and of beta-lac (present in knockout vs. wild-type mice) (Millan *et al*., 2008). The depressant effects of TYR ($n = 5$), β-PEA ($n = 7$) and AMPH ($n = 4$) were still present in these mice (Figure 3Aa, b). The TAs-induced depression was similar to that observed in C57BL6 wild-type mice both for the kinetics and the percentages of reduction obtained (Figure 3Ab). Specifically in TA_1 KO mice, β-PEA (100 µM) and TYR (100 µM) reduced the quinpirole-induced current to 25.5 ± 9.8% ($n = 7$) and to 56.6 ± 11.8% ($n = 5$) of control values, respectively, whereas in wild-type mice corresponding doses inhibited GIRK currents to 49.5 ± 5.1% ($n = 18$, $P < 0.05$) and 48.1 ± 9.3% ($n = 10$, $P > 0.05$) of control.

In addition, in normal mice, the selective agonist of TA_1 *O*-PIT (Hart *et al*., 2006), bath applied at a concentration of 100 µM ($n = 6$) for more than 5 min, was not able to mimic the reduction of the D_2 receptor-mediated responses induced by the TAs (Figure 3B). β-PEA 100 µM induced a reduction of 49.5 ± 5.1% whereas that to *O*-PIT 100 µM was 10.3 ± 2.9% (Figure 3B).

The effects of TYR and β-PEA are not caused by activation of σ receptors
The next step was to investigate whether the inhibitory effect induced by TAs on quinpirole-activated GIRK currents was caused by the activation of σ receptors. Recently, it was shown that both β-PEA and TYR can bind and activate σ receptors (σ1 and σ2), displaying the highest affinities for the σ2 receptors (Fontanilla *et al*., 2009). We tested this possibility by superfusing β-PEA with a cocktail of σ1 and σ2 antagonists. The concomitant perfusion of the σ2 antagonist, SM-21 (30 µM for 10 min) with the selective σ1 antagonist, BD1047 (30 µM for 10 min) had no effect on the depressant effect of β-PEA (Figure 3C) on the quinpirole-induced current ($n = 4$, 46.3 ± 6.1% in presence of antagonists vs. 49.5 ± 5.1% in control conditions, $P > 0.05$).
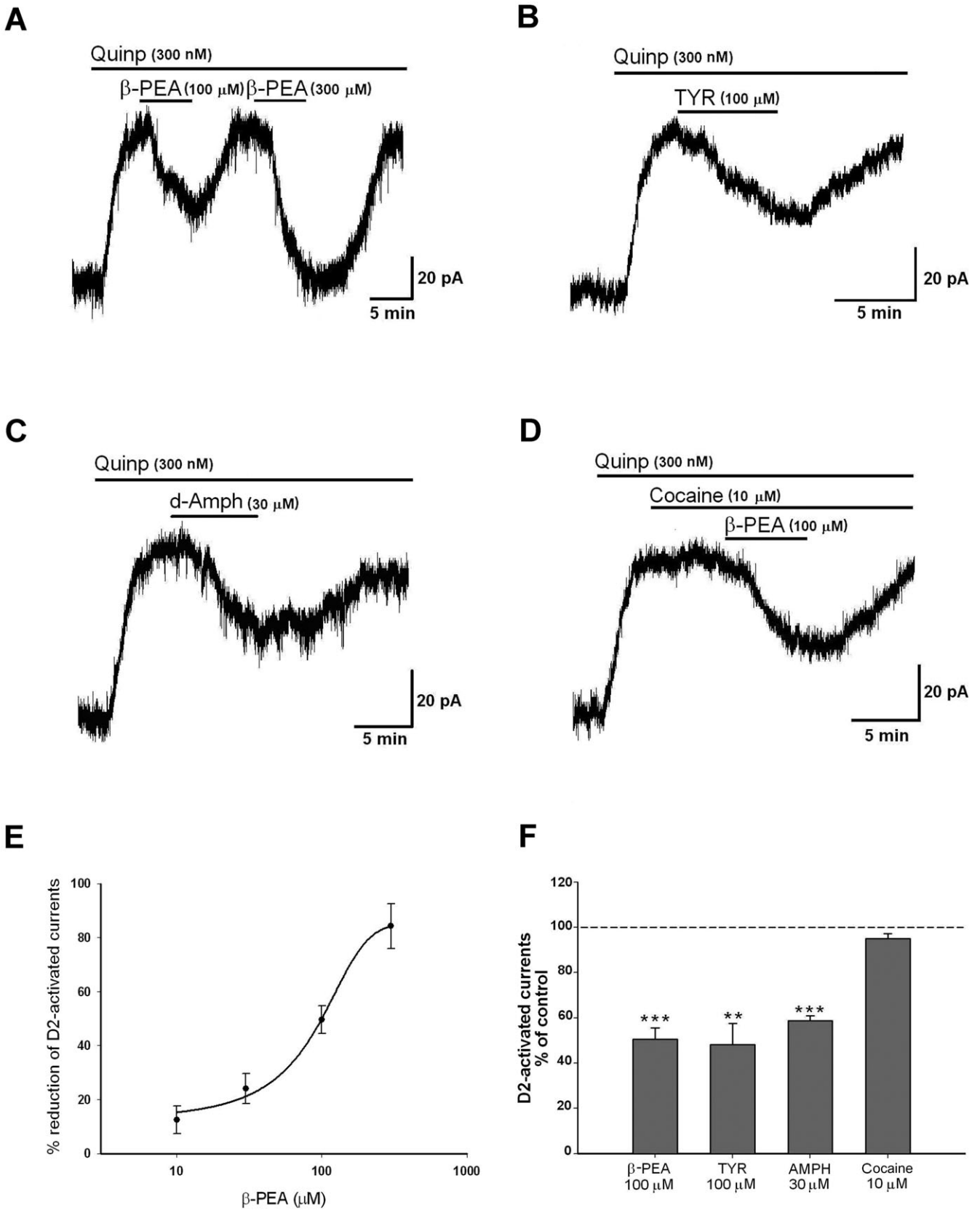
Figure 1 (A) Representative recordings showing the inhibitory effect of β-phenylethylamine (β-PEA) (A) and tyramine (TYR) (B) on the quinpirole-induced current. Effects were mimicked by d-amphetamine (AMPH) (C) but not by cocaine (D). Of note, cocaine does not modify the action of β-PEA. From the concentration-response curve shown in (E), the value of the IC_{50} was 82.9 ± 9.6 μM for β-PEA ($n = 6$–8 cells for each concentration). (F) The columns indicate the normalized residual quinpirole-induced current (% of control) observed in the presence of β-PEA (100 μM, $n = 17$, $P < 0.001$), TYR (100 μM, $n = 10$, $P < 0.005$), AMPH (30 μM, $n = 7$, $P < 0.001$) and cocaine (10 μM, $n = 4$, $P > 0.05$).
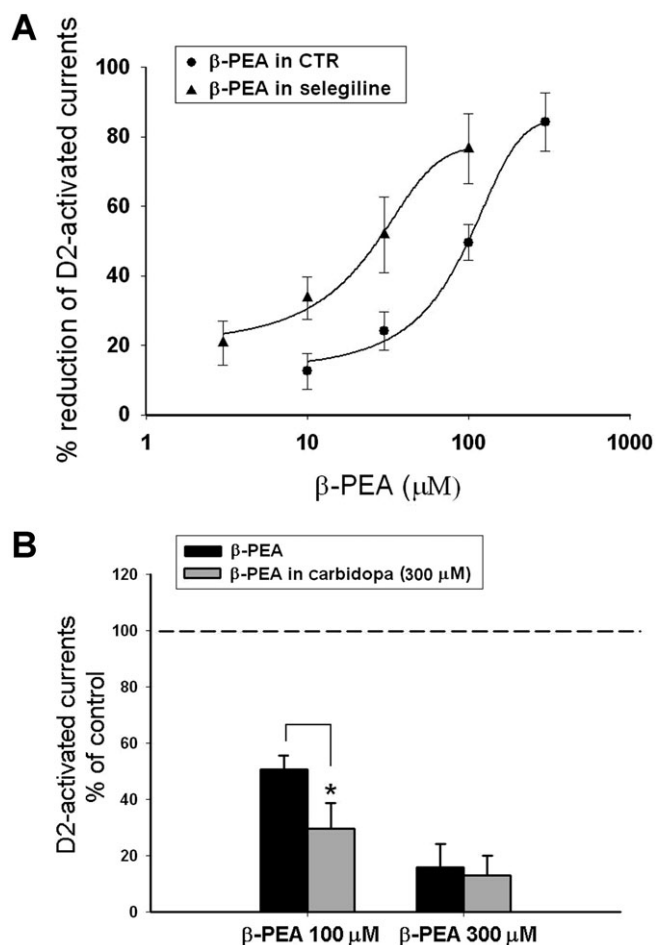
Figure 2 Effects of β-phenylethylamine (β-PEA) in the presence of enzymatic inhibition of catabolic and synthesizing pathways. (A) The concentration-response curve of β-PEA was shifted to the left in the presence of the monoamine oxidase (MAO) B inhibitor selegiline (100 nM) ($n = 7$ cells per concentration). (B) The L-amino acid decarboxylase (AADC) inhibitor carbidopa did not modify the effect of 300 μM β-PEA but increased the responses to 100 μM β-PEA. The enhancing effect of carbidopa at the lower concentration of β-PEA (100 μM) could be because of a drop in the endogenous level of amines caused by this AADC inhibitor and thus, a more marked effect of the exogenously applied trace amine is detected (*$P < 0.05$; the results depicted by the columns were obtained from $n = 7$ experiments).

TAs reduce D_2-activated GIRK current through a G-protein independent mechanism

In order to ascertain whether the D_2 receptor-mediated outward current is negatively modulated by TAs, we generated current–voltage plots in control conditions during the perfusion of quinpirole and quinpirole plus β-PEA. As expected for an involvement of GIRK the crossing of the resultant net currents was −101 mV (Figure 4A) (Lacey *et al.*, 1987).

To further characterize the mechanism by which TAs depress the D_2 receptor-activated GIRK current we analysed the potential involvement of G-protein signalling pathways. Hence, we loaded dopaminergic neurones with the non-hydrolyzable GTP analogue, GTP-γ-S trilithium salt (GTPγS, 1 mM). It has been demonstrated previously that intracellular GTPγS diffusion in dopaminergic neurones leads to activation

of a sustained outward current because of progressive and permanent activation of $G_{i/o}$ proteins (Lacey *et al.*, 1987). Under these conditions, the stimulation of D_2 receptors results in an irreversible outward response suggesting a persistent GIRK opening (Figure 4Ba). The mean D_2 receptor-mediated outward current was 183 ± 32 pA ($n = 5$) whereas the subsequent application of β-PEA (100 μM, 5 min) induced a sustained inward current of 95 ± 18 pA ($n = 5$), which was reversible (wash out 15 min). Additional experiments were performed in GTPγS-treated neurones using the GABA$_B$ agonist, baclofen (10 μM), which activates the same current as dopamine (Lacey *et al.*, 1988). Under these conditions, the outward current of 187 ± 22 pA ($n = 3$), irreversibly activated by baclofen, was transiently depressed by β-PEA (100 μM, 5 min; not illustrated).

In contrast, the superfusion of the mGluR agonist DHPG (10 μM), superimposed on the quinpirole-induced outward current, caused an inward current (Guatteo *et al.*, 1999) that did not wash out, implying that other G-protein-mediated events (presumably Gq, see Discussion, Cho *et al.*, 2005) could be still irreversibly activated when GTPγS is infused into the cells (Figure 4Bb).

TAs do not affect GIRK currents expressed in oocytes

In order to test the effect of β-PEA on Kir3.2 potassium channels (GIRK2) activity, RNA coding for Kir3.2 channels was injected into *Xenopus* laevis oocytes and the resulting currents were recorded under TEVC configuration. Figure 5A shows representative current traces recorded from an oocyte expressing Kir3.2 channels before (CTRL) and after the superfusion with a solution containing β-PEA (200 μM). The application of β-PEA did not affect the Kir3.2 potassium current recorded at −120 mV. (Figure 5A, C). In particular, the Kir3.2 potassium current in control conditions was 580 ± 140 nA ($n = 12$), whereas after β-PEA application it was 610 ± 210 nA ($n = 12$, $P > 0.05$). In contrast, the application of Ba^{2+} (200 μM), a Kir channel blocker, inhibited the current amplitudes readily (Figure 5B, C). Therefore, to determine whether β-PEA only modulates the dopamine-evoked potassium current, Kir3.2 were co-expressed with D_2 receptors and the effect of β-PEA on the dopamine-evoked Kir3.2 potassium current was tested. Figure 5D shows representative whole-cell current traces recorded from an oocyte co-expressing Kir3.2 and D_2 receptors under basal condition (CTRL), during the superfusion of dopamine (20 μM), β-PEA (200 μM) and after wash-out (W/O). Dopamine (20 μM) increased basal Kir3.2 potassium currents (1130 ± 300 nA, $n = 10$) ~1.5-fold (1700 ± 300 nA, $n = 10$, $P < 0.001$) in a reversible manner (Werner *et al.*, 1996), whereas β-PEA did not affect dopamine-evoked currents (Figure 5D, F). Indeed, after the application of β-PEA, the dopamine-evoked current was 1500 ± 320 nA ($n = 10$). Similar negative effects were obtained with a superfusion of TYR (200 μM; not shown).

Discussion

Depressant effects of TAs on D_2 receptor-mediated responses

The present results demonstrate that the prototypical TAs, β-PEA and TYR, both concentration-dependently depressed
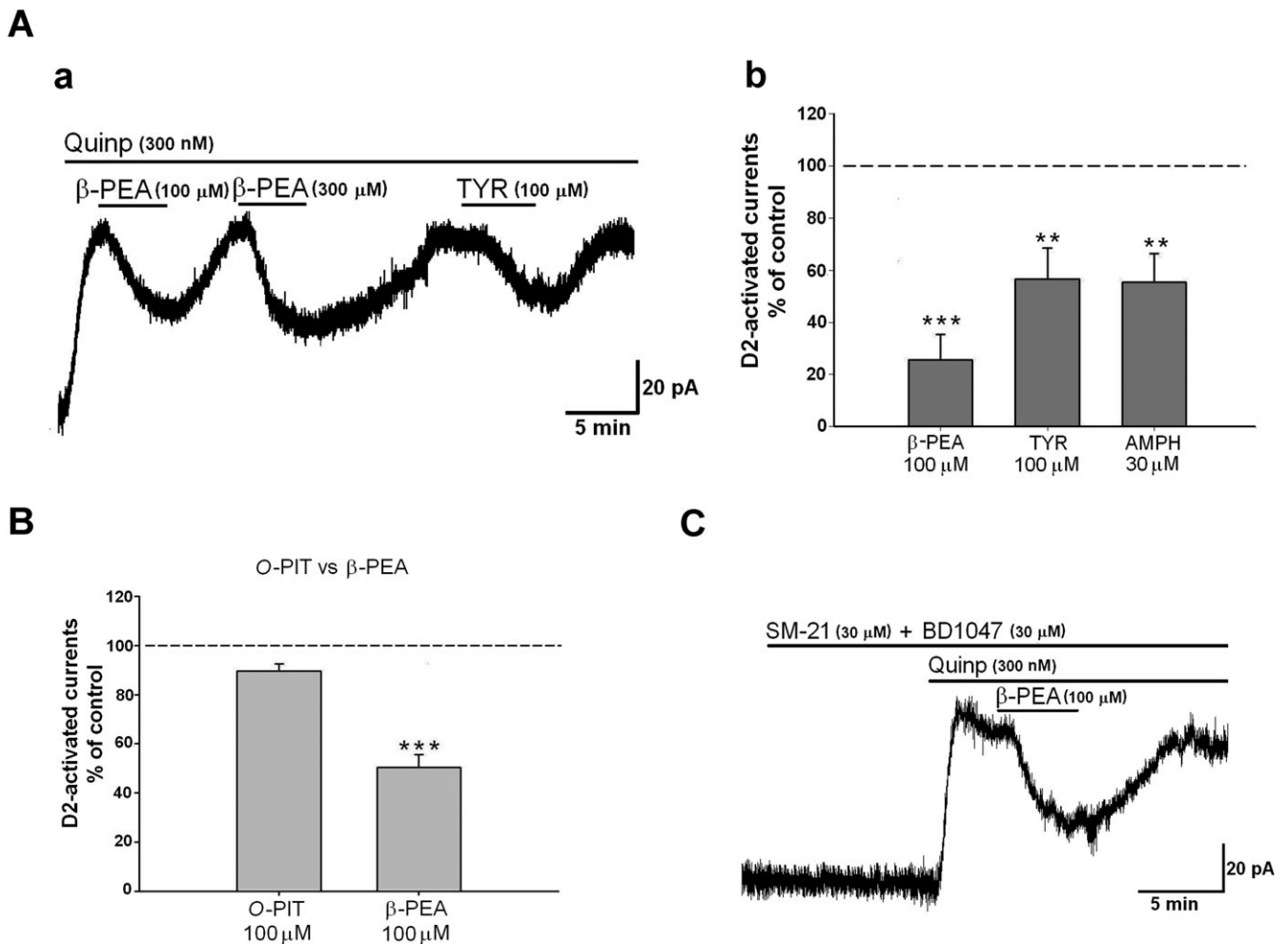
A

a

b

B

C

Figure 3 (A) (a) Representative trace showing that a consistent and reproducible inhibition of the quinpirole-induced outward current was caused by β-phenylethylamine (β-PEA; 100–300 μM) and tyramine (TYR; 100 μM) in a dopaminergic neurone obtained from a TA$_1$ knockout mice. (b) A similar depression was caused by d-amphetamine (AMPH) (30 μM). (B) The histogram shows that the specific TA$_1$ agonist o-phenyl-3-iodotyramine (O-PIT; 100 μM) had no effect on the D$_2$ receptor-mediated outward response. In contrast to β-PEA ($P < 0.001$ for O-PIT vs. β-PEA), an infusion of O-PIT (100 μM) induced only a small but non-significant reduction of G protein-gated inward rectifier K$^+$ channel (GIRK) currents ($n = 6$, $P > 0.05$). (C) Representative trace showing that β-PEA still inhibits the quinpirole-induced outward current in the presence of the σ2 antagonist, SM-21 (30 μM) and the σ1 antagonist, BD1047 (30 μM).

the D$_2$ receptor-mediated outward current in dopaminergic neurones. Inasmuch as the effects of β-PEA were particularly robust and consistent, we focused our attention on this agent which expressed its actions with an IC$_{50}$ of around 80 μM. This concentration is higher than that which has generally been detected in the brain tissue homogenates (Boulton, 1976; Berry, 2004; Grandy, 2007), but is still likely to be physiologically relevant in view of: (i) the extremely rapid turnover of β-PEA and other TAs (which have a half-life of approximately 30 s); and (ii), the likelihood that free, synaptic, extracellular levels of TA are, at least transiently, much higher upon their release (Durden and Philips, 1980). Moreover, it must be emphasized that the penetration of TAs into tissue slices is limited by the diffusion barrier and degradation/sequestering mechanisms: hence, a high amount needs to be applied in *in vitro* studies in order to achieve a sufficient concentration at the effective site of action. By analogy, dopamine only affects neuronal activity when superfused in the micromolar range (Lacey *et al*., 1987; Suppes and

Pinnock, 1987) at concentrations far higher than those found in tissue or in the extracellular fluid, but corresponding to concentrations present and active at the synaptic level (Ford *et al*., 2009). It should also be pointed out that cerebral levels of TAs are not constant inasmuch as pathological states such as Rett syndrome and phenylketonuria are characterized by low (Satoi *et al*., 2000) and high (Perry, 1962) brain levels of β-PEA, respectively. Furthermore, the dose-response curve for β-PEA was shifted to the left (4.2-fold) in the presence of the MAO B inhibitor, selegiline, consistent with the notion that the functional activity of this catabolic enzyme is important for regulating the turnover and, hence, the effects of TAs on neurones. Accordingly, in certain pathophysiological states, and following functional, genetic or pharmacological manipulations that reduce MAO activity, the availability of TAs will be enhanced and their neuronal actions amplified.

Based on experiments with the AADC inhibitor, carbidopa, which by reducing the formation of dopamine and noradrenaline blunts their TA-induced release (Geracitano *et al*.,
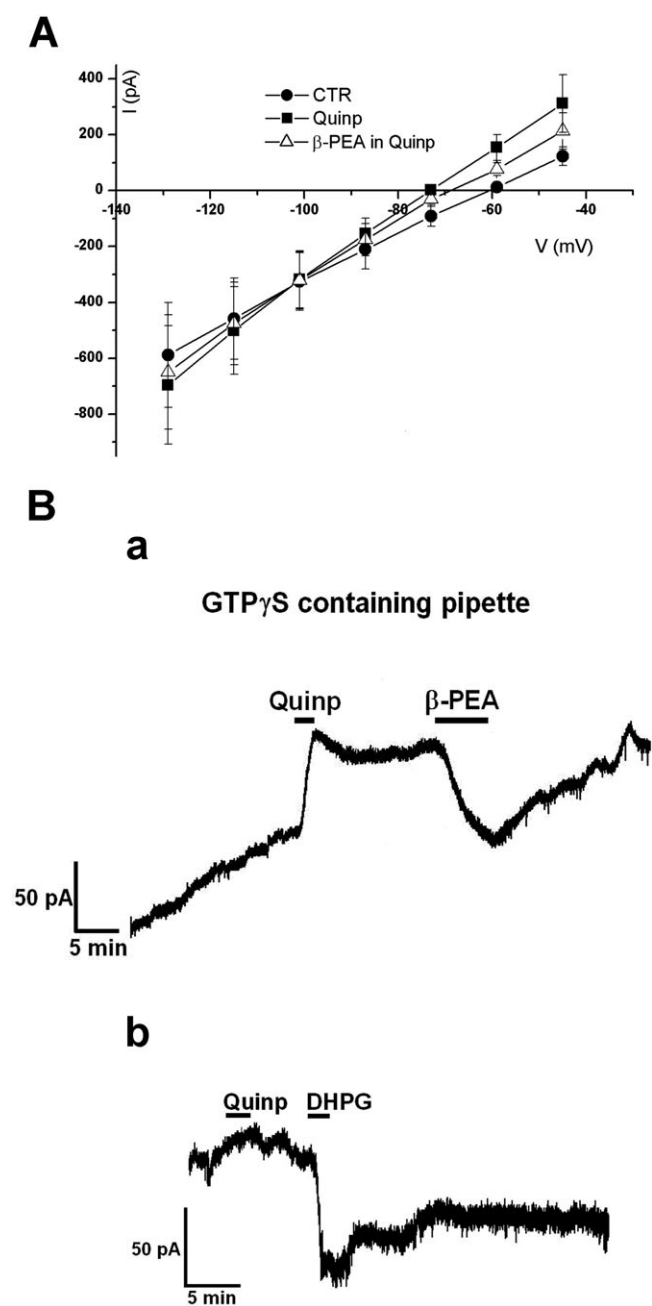
Figure 4 Trace amines reduced the D_2 receptor-activated G protein-gated inward rectifier K$^+$ channel (GIRK) current through a G-protein independent mechanism. (A) The current–voltage relationships performed by voltage steps in control conditions, in quinpirole and in quinpirole plus β-phenylethylamine (β-PEA; 100 μM) demonstrate that the quinpirole-induced outward currents and the β-PEA-induced inward current (quinp plus β-PEA) all reversed at –101 mV. Note that the point of intersection for the currents in the three different conditions above is close to K$^+$ reversal potentials. Each point represents the mean ± SEM ($n = 4$). (B) (a) Representative current trace showing that the intracellular diffusion of GTPγS in a dopaminergic neurone caused a progressive outward current that was augmented by quinpirole (300 nM). Under this condition a relative short application of the D_2 receptor agonist caused a sustained outward current that was transiently depressed by β-PEA (100 μM). (b) Noteworthy, whereas the inward current caused by β-PEA washed-out, the one induced by the mGluR 1 agonist DHPG (10 μM) was sustained indicating the involvement of G-protein in the latter case.

2004), it appears that the effects of β-PEA and TYR were not indirectly mediated via extracellular increases in levels of these two catecholamines. In line with these findings, it has been shown that an intraventricular injection of TYR elicits locomotor activity in rats that had been pretreated with reserpine or α-methyl-p-tyrosine to exclude the possibility of indirect receptor stimulation following catecholamine release (Stoof *et al.*, 1976). Moreover, other effects of TA exerted independently of dopamine and noradrenaline (Paterson, 1993) have also been described.

Noteworthy, in the present study, the inhibitory effect of TAs on the D_2 receptor-mediated outward current was mimicked by the dopamine-releasing drug AMPH but not by cocaine, a dopamine uptake blocker. Thus, the persistence of the modulatory effect induced by the TAs in the presence cocaine suggests that dopamine transporters (DATs) are not involved in this effect.

Absence of mechanisms mediated by TA_1 receptors
Interestingly, the depressant actions of β-PEA and TYR were present in transgenic mice in which the TA_1 receptor had been genetically deleted (TA_1 null mice) (De Groote *et al.*, 2007; Millan *et al.*, 2008). Underpinning these observations, the selective TA_1 agonist, *O*-PIT (Hart *et al.*, 2006) proved to be ineffective in depressing D_2 receptor-mediated responses, despite the fact that several other functional responses to this agent, like the induction of hypothermia, are abolished in TA_1 knock-out mice (above citations).

Collectively, these data support the idea that TA_1 receptors are not involved in the depression of the D_2 receptor-mediated responses of SNpc dopaminergic neurones induced by β-PEA and TYR observed in the present study.

Of note, TA_1 is distributed throughout cerebral populations of monoaminergic neurones and is co-localized with DAT in a subset of dopaminergic neurones in mouse and rhesus monkey SNpc (Xie *et al.*, 2007). A mutual functional interaction between TAs and dopaminergic transmission has been described (Lindemann *et al.*, 2008; Sotnikova *et al.*, 2008; Xie *et al.*, 2008), in that D_2-autoreceptor activation appears to reduce TA_1 signalling (Xie *et al.*, 2007) that, contrarily, is increased by the co-expression of DAT with TA_1 in HEK293 cells (Miller *et al.*, 2005). Reciprocally, both TA_1 and D_2 receptors modulate DAT activity but exert opposite effects (Xie *et al.*, 2008). Notably, the neuronal hyperpolarization produced in dopaminergic mesencephalic neurones by TA-induced release of DA via DAT was impaired in TA_1 KO mice (Lindemann *et al.*, 2008). Moreover, it has been recently shown that TA_1 modulates D_2 receptor activity, fine-tuning their desensitization rate and the potency of agonists (Bradaia *et al.*, 2009). It should be mentioned that, in our experimental conditions, the outward current induced by long-lasting application of quinpirole (300 nM) did not desensitize, so this phenomenon was not monitored. Recently, it was proposed that TYR inhibits the firing activity of dopaminergic neurones and induces the opening of inwardly rectifying K$^+$ channels through a TA_1-dependent mechanism (Bradaia *et al.*, 2009). These data are not consistent with our previous results showing that the inhibitory effects of TYR and β-PEA are indirect (mediated by
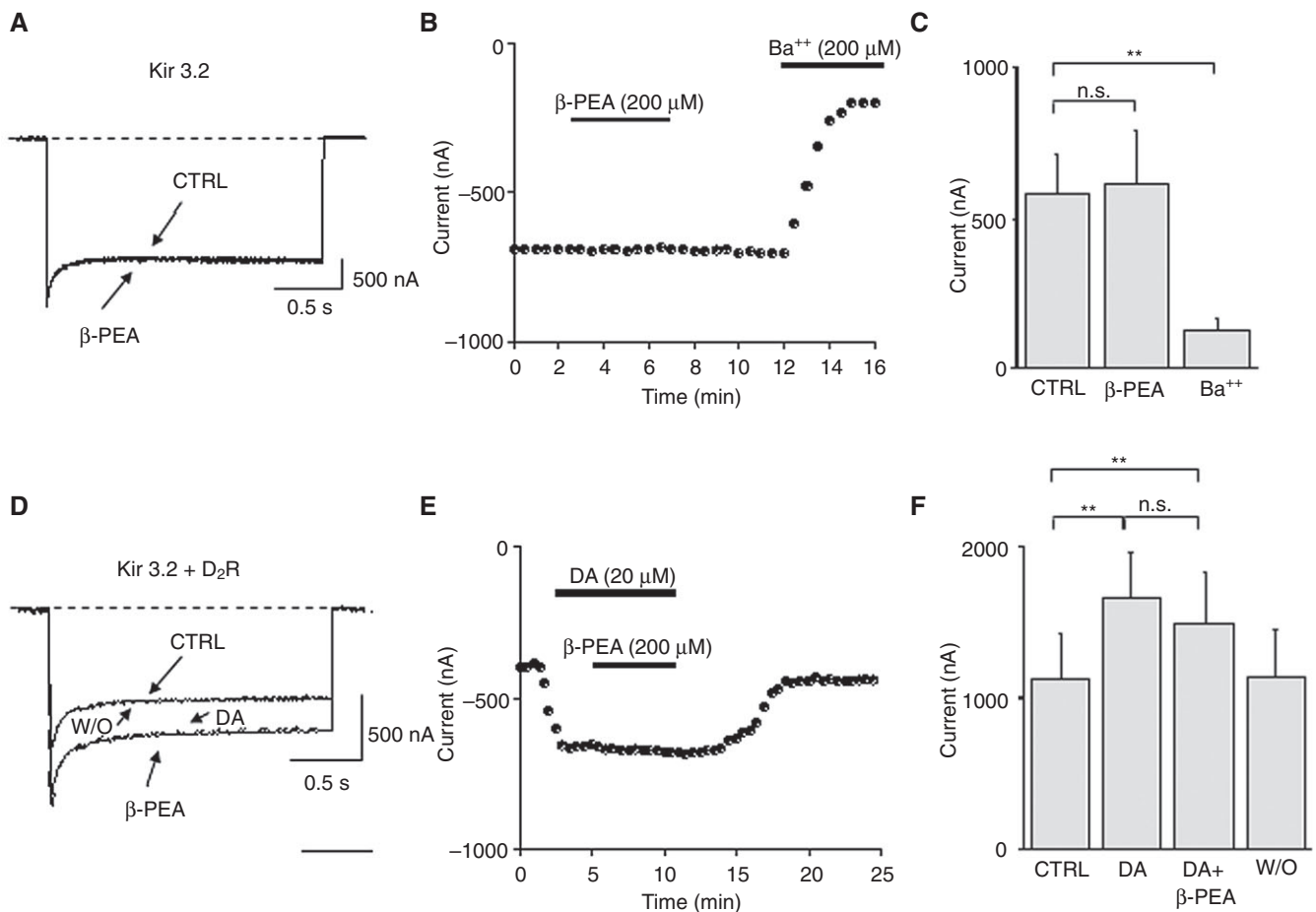
Figure 5 Effect of β-phenylethylamine (β-PEA) on both the basal and dopamine-evoked Kir3.2 potassium currents in heterologous expression systems. (A) Representative whole-cell current traces recorded from a *Xenopus* oocyte expressing Kir3.2 channels before (CTRL) and after superfusion of β-PEA (200 μM). Currents were elicited by hyperpolarizing steps to –120 mV for 2 s from a holding potential of –10 mV. The dashed line indicates zero current. (B) Time-course for the effect of β-PEA (200 μM) and Ba^{2+} (200 μM) on current amplitudes recorded from oocytes expressing Kir3.2 channels. The horizontal bar indicates the time of β-PEA and Ba^{2+} application. (C) Histogram showing the mean current amplitudes recorded at –120 mV from oocytes expressing Kir3.2 channels before and after the superfusion of β-PEA and Ba^{2+}. (D) Representative whole-cell current traces recorded from an oocyte co-expressing Kir3.2 and D_2 receptors showing the basal Kir3.2 current (CTRL), the dopamine-evoked Kir3.2 current, the effect of β-PEA (200 μM) application and wash-out (W/O). (E) Time-course of current amplitudes recorded before and after the application of dopamine (20 μM) and β-PEA (200 μM), from an oocyte co-expressing Kir3.2 and D_2 receptors. The horizontal bars indicate the time of the dopamine and β-PEA application. (F) Histogram showing the mean Kir3.2 current amplitudes recorded at –120 mV under control conditions, during the application of dopamine (20 μM), β-PEA (200 μM) and after wash-out of dopamine (W/O). These results indicate that β-PEA does not affect either basal or dopamine-evoked Kir3.2 channel activity. The data are mean ± SEM of six to eight cells. (***$P < 0.001$, Student's *t*-test).

dopamine); they were shown to be abolished by sulpiride and greatly reduced by depletion of dopamine (carbidopa-induced) (Geracitano *et al.*, 2004). Notably, indirect dopamine-mediated effects of TYR have also been reported in the subthalamic nucleus (Zhu *et al.*, 2007). Although species differences (rat versus mice) could partially account for the divergent results, the clear-cut D_2 receptor-mediated effects of β-PEA in TA_1 KO mice clearly differ from the recent observations of Bradaia *et al.* (2009).

Absence of mechanisms mediated by σ receptors
σ Receptors have been reported to inhibit diverse classes of K^+ and Ca^{2+} ion channels through direct protein-protein interactions with a G-protein independent mechanism not related to protein kinases (Lupardus *et al.*, 2000; Fontanilla *et al.*, 2009).

A reduction of GIRK-mediated response has been reported with σ agonists (Kobayashi *et al.*, 2006; Monassier *et al.*, 2007) both in the heart and also in catecholaminergic neurones in the CNS (Nguyen *et al.*, 1998). As it was recently shown that both β-PEA and TYR activate σ receptors (Fontanilla *et al.*, 2009), we used selective antagonists of σ1 and σ2 subtypes to verify if they are involved in their depressant effect on the D_2 receptor-mediated outward current. Inasmuch as they were ineffective, these results suggest that TAs do not stimulate σ receptors to modulate D_2 receptor-activated responses in the dopaminergic cells.

Cellular mechanisms of action
It is known that stimulation of D_2 autoreceptors activates $G_{i/o}$ proteins to open inward rectifier potassium channels. This

has again been supported by the experiments in which the reversal potential of the D_2 receptor-mediated outward current is compatible with potassium equilibrium (Lacey *et al*., 1987, 1988). Moreover, the involvement of the $G_{i/o}$ proteins has been confirmed by single-electrode voltage clamp experiments that permitted a successful intracellular diffusion of the irreversible non-hydrolysable GTP analogue GTPγS. Thus, the dissociated $G_{\beta\gamma}$ subunits permanently engaged the receptor-operated K^+ channels (Lacey *et al*., 1987). It is intriguing, then, that the GTPγS experiments conduced herein suggested a lack of G-protein involvement in the depressant effect of TAs. Based on the action of GTPγS – intracellularly dialysed using a patch-clamp technique – we previously reported that G-proteins are involved in the depression of GABA$_B$-mediated outward currents by TAs (Federici *et al*., 2005). However, in retrospect, there may have been a misinterpretation of our data because of excessive dialysis of the intracellular milieu during prolonged patch-clamp recording. It is likely that an excessive dialysis of the cytoplasmatic content, because of the use of the patch-clamp technique, caused a subsequent rundown of GABA$_B$-mediated responses, which was erroneously considered to represent an irreversible inhibitory effect of TAs. This is also supported by newer experiments using baclofen in GTPγS-treated neurones (see Results). Hence, we suggest that during prolonged electrophysiological experiments requiring GTPγS dialysis and designed to examine G-protein-mediated effects, it is preferable to use – as herein – sharp microelectrode techniques rather than conventional patch-clamp recordings. The current–voltage plots performed in the presence of quinpirole and β-PEA demonstrated that the TA-reduced current reversed at the potassium equilibrium point, confirming that TAs reduce a GIRK-activated channel. Surprisingly, the experiments performed in oocytes suggest that TAs act neither at D_2 receptors nor on GIRK channels themselves leading to the hypothesis that a more complex sequence of events leads to the reduction of the D_2 receptor-activated GIRK currents by TAs. However, as the Gβγ subunit combination expressed by oocytes is not known, it is possible that they differ from dopaminergic cells of the substantia nigra and are therefore insensitive to modulation by TAs. Thus, taken together, the present data suggest that TAs diminish quinpirole-induced outward currents by a molecular mechanism that needs to be more fully elucidated in future studies. However, a comparable depressant effect of TA has also been observed on GIRK currents activated by GABA$_B$ receptors (Federici *et al*., 2005 and the present data) and, as the same type of GIRK channels are activated by D_2 and GABA$_B$ receptors (Lacey *et al*., 1988), a similar mechanism is probably involved in the modulation, by β-PEA and other TAs, of the GIRKs induced by both D_2 and GABA$_B$.

It is known that GIRK currents can be inhibited by the activation of receptors coupled to $G_{q/11}$ leading, through the induction of phospholipase C (PLC), to depletion of phosphatidylinositol 2,4-bisphosphate (PIP$_2$) (Cho *et al*., 2005). However, the experiments performed using GTPγS in which mGluR1 (DHPG)/$G_{q/11}$–mediated responses were found to be irreversible, make it unlikely that such a mechanism is involved in the *reversible* inhibition of D_2 receptor-activated GIRK currents induced by TAs.

Finally, based on the lack of effects of TAs in D_2 receptor- and GIRK-expressing oocytes there is a real possibility that specific neuronal sites exist at which TA could act to affect D_2 receptor/G-protein-operated GIRK channel activity. Hence, TAs could modulate neuronal GIRK activity by altering the efficiency of Gβγ coupling to the channel. In line with this notion, recent evidence indicates that – apart from their cognate heterotrimeric G proteins – D_2 receptors interact with a large number of dopamine receptor interacting proteins ('DRIPs') involved in scaffolding, trafficking and coupling to intracellular effectors (Kabbani and Levenson, 2007). Therefore, it is conceivable that TAs hamper the interaction between receptors and DRIPs, thereby leading to an inhibition of D_2 receptor-activated GIRK currents.

Functional considerations

It is possible that the negative regulation of D_2 autoreceptor function by TA contributes to the regulation of the normal basal activity of dopaminergic cells, and it might also render them more excitable. An excitatory effect of TAs on cortical and subthalamic neurones has been described previously (Zhu *et al*., 2007; Kitamura *et al*., 2008) and it was demonstrated that β-PEA inhibits K^+ currents in neocortical neurones of the rat (Kitamura *et al*., 2008). TA-induced blunting of D_2 autoreceptor inhibition has potentially important implications for the control of movement, reward, mood, motor function and cognition and, consequently, may be pertinent to the aetiology and treatment of diseases in which perturbed dopaminergic transmission has been implicated, such as schizophrenia, drug abuse, depression, Parkinson's disease and attention deficit hyperactive disorders (Boulton, 1980; Davis and Boulton, 1994; Bergman *et al*., 2001; Millan, 2006; Sotnikova *et al*., 2008).

Concluding remarks

The present data demonstrate that the prototypical TAs, β-PEA and TYR, both concentration-dependently suppress D_2 receptor-mediated GIRK currents in SNpc localized dopaminergic neurones, actions expressed neither via TA$_1$ receptors nor via σ1 receptors, and in a G-protein independent fashion. The underlying molecular substrates remain to be determined. However, they may involve intracellular modulation by TAs of protein-protein interactions downstream of D_2 receptors thereby modifying their influence on GIRKs – and other cellular effectors. In any event, these data add further weight to the notion that the actions of TA are by no means limited to modulation of catecholamine release and that they directly influence the activity of monoaminergic and, in particular, dopaminergic pathways.

Conflict of interest

None.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Representative current trace showing that the intracellular diffusion of GTPγS in a dopaminergic neurone caused a progressive outward current. Under this condition, a relative short application of the GABA_B receptor agonist, baclofen (10 μM), caused a sustained outward current that was transiently depressed by β-phenylethylamine (β-PEA) (100 μM).

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