Constitutive activity of dopamine receptor type 1 (D1R) increases Ca\textsubscript{v}2.2 currents in PFC neurons

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Alterations in dopamine receptor type 1 (D1R) density are associated with cognitive deficits of aging and schizophrenia. In the prefrontal cortex (PFC), D1R plays a critical role in the regulation of working memory, which is impaired in these cognitive deficit states, but the cellular events triggered by changes in D1R expression remain unknown. A previous report demonstrated that interaction between voltage-gated calcium channel type 2.2 (Ca\textsubscript{v}2.2) and D1R stimulates Ca\textsubscript{v}2.2 postsynaptic surface location in medial PFC pyramidal neurons. Here, we show that in addition to the occurrence of the physical receptor-channel interaction, constitutive D1R activity mediates up-regulation of functional Ca\textsubscript{v}2.2 surface density. We performed patch-clamp experiments on transfected HEK293T cells and wild-type C57BL/6 mouse brain slices, as well as imaging experiments and cAMP measurements. We found that D1R coexpression led to \textasciitilde 60% increase in Ca\textsubscript{v}2.2 currents in HEK293T cells. This effect was blocked by preincubation with a D1/D5R inverse agonist, chlorpromazine, and by replacing D1R with a D1R mutant lacking constitutive activity. Moreover, D1R-induced increase in Ca\textsubscript{v}2.2 currents required basally active Gs protein, as well as D1R-Ca\textsubscript{v}2.2 interaction. In mice, intraperitoneal administration of chlorpromazine reduced native Ca\textsubscript{v}2.2 currents' sensitivity to \textomega-conotoxin-GVIA and their size by \textasciitilde 49% in layer V/VI pyramidal neurons from medial PFC, indicating a selective effect on Ca\textsubscript{v}2.2. Additionally, we found that reducing D1/D5R constitutive activity correlates with a decrease in the agonist-induced D1/D5R inhibitory effect on native Ca\textsubscript{v}2.2 currents. Our results could be interpreted as a stimulatory effect of D1R constitutive activity on the number of Ca\textsubscript{v}2.2 channels available for dopamine-mediated modulation. Our results contribute to the understanding of the physiological role of D1R constitutive activity and may explain the noncanonical postsynaptic distribution of functional Ca\textsubscript{v}2.2 in PFC neurons.

Introduction

Dopamine receptors constitute a family of G protein-coupled receptors (GPCRs) that are widely expressed in the brain and contribute to diverse neuronal functions. In particular, the D1-like receptor subfamily, which includes the dopamine receptor type 1 (D1R) and D5R, plays a key role in locomotor activity (Svensson et al., 2017) and cognitive and social behaviors (Cools, 2008; Homberg et al., 2016). The effects of D1R/D5R stimulation by agonists have been thoroughly studied in behavioral tests in rodents (Zahrt et al., 1997; Stubendorff et al., 2019) and primates (Sawaguchi and Goldman-Rakic, 1991; Arnsten et al., 2015). Moreover, there is strong evidence that different physiological and pathological states greatly correlate with changes in D1R/D5R expression levels. For instance, in bird neurons, D1-like receptor mRNA increases after working memory training, while D2R mRNA remains unchanged (Herold et al., 2012). In human brains, alterations in D1/D5R receptor density and sensitivity to dopamine are associated with cognitive deficits of aging and schizophrenia (Wang et al., 1998; Thompson et al., 2014). Dopamine binding to D1/D5R receptors also changes after working memory training in humans (McNab et al., 2009). While many studies have examined these behavioral effects related to alterations in D1/D5R receptor properties, the downstream cellular events triggered by these changes remain unknown.

D1/D5R regulate working memory processes by acting mainly in the prefrontal cortex (PFC) in rodents and primates (Jones, 2002; Williams and Castner, 2006), and their expression levels in this brain area are dramatically impaired in cognitive deficit states (Goldman-Rakic et al., 2004). In particular, several studies have established a relationship between improved cognitive test performance in mice and higher levels of D1R protein specifically in the medial PFC (mPFC; Kolata et al., 2010; Wass et al., 2013, 2018). A recent report found that the origin of this D1R increase is related to the DRiP78 chaperonin (Wass et al., 2018), but their cellular targets are unknown.
Meanwhile, DIR physically interacts with voltage-gated calcium channel type 2.2 (CaV2.2) and increases channel protein density at postsynaptic sites of mPFC pyramidal neurons (Kisilevsky et al., 2008). Higher activity-mediated calcium influx could thus represent a cellular consequence of increased DIR expression levels. Here, we aimed to understand the underlying mechanisms and functional output of CaV2.2 modulation by DIR.

A relevant feature of DIR that could be involved in the increase in CaV2.2 in the plasma membrane is the receptor’s constitutive activity. The importance of GPCR agonist independent activity is becoming increasingly clear (Costa and Cotecchia, 2005; Meye et al., 2015). For instance, our laboratory has demonstrated that constitutive activity of two different GPCRs has a great impact on calcium channel function (López Soto et al., 2015; Agosti et al., 2017; Mustafá et al., 2017; Martínez Damonte et al., 2018). For D1/D5R, there is an abundance of in vitro reports demonstrating a basal increase in Gs activity in several systems (Plouffe et al., 2010; Zhang et al., 2014; Zhang et al., 2015). Our studies extend the role of DIR constitutive activity in a physiologically relevant event: the stimulation of mPFC native and recombinant CaV2.2 currents by DIR expression. Moreover, we have combined the study of dopamine-mediated activity with the exploration of the role of DIR constitutive activity to modulate CaV2.2 to propose a compelling model that expands our understanding of DIR function in the brain.

Materials and methods

Animals and ethical approval

All experiments in this study received approval from the ethical committee of the Multidisciplinary Institute of Cell Biology (IMBICE), in strict accordance with the recommendations of the U.S. Guide for the Care and Use of Laboratory Animals of the National Research Council (reference number from the Ethical Committee of IMBICE; #12-03-19). All possible actions to minimize suffering were taken. Experiments were conducted on C57BL/6 WT mice of both sexes. Mice were bred and housed at the IMBICE animal facility with a 12-h light/dark cycle, controlled food and water. Animals and ethical approval were taken. Experiments were conducted on C57BL/6 WT mice of both sexes. Mice were bred and housed at the IMBICE animal facility with a 12-h light/dark cycle, controlled room temperature (22°C ± 2°C), and ad libitum access to food and water.

Animal treatment and PFC slice preparations

A total of 18 mice were used in this study (vehicle, n = 11; chlorpromazine-treated, n = 7). 4–6-wk-old WT mice were treated with vehicle (saline solution NaCl 0.9%) or chlorpromazine (1 mg/kg) through two intraperitoneal (IP) injections (0.1 ml/10 g) 24 h and 1 h before sacrifice. Mice were anesthetized with isoflurane (2%) and immediately decapitated. Brains were quickly removed and immersed in ice-cold 95% O2 and 5% CO2-equilibrated cutting solution containing (in mM) 110 choline chloride, 25 glucose, 25 NaHCO3, 7 MgCl2, 1.6 ascorbic acid, 3.1 sodium pyruvate, 2.5 KCl, 1.25 NaH2PO4, and 0.5 CaCl2, pH 7.4, with CsOH. Coronal brain slices including the mPFC (−300 µM, 1.5–2.5 mm anterior to bregma) were obtained using a vibratory tissue slicer (PELCO easySlicer; #11000; Ted Pella Inc.) and then transferred to an incubation chamber filled with 95% O2, 5% CO2–equilibrated artificial cerebrospinal fluid (aCSF) containing (in mM) 124 NaCl, 26.2 NaHCO3, 11 glucose, 2.5 KCl, 2.5 CaCl2, 1.3 MgCl2, and 1 NaH2PO4, pH 7.4, with HCl. Slices were maintained at 37°C for 15 min and left to recover at room temperature (~24°C) for 30 min before recordings.

Cell culture and transient transfections

HEK293T cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; #D5030, Gibco; Thermo-Fisher) with 10% FBS (Internegocios A.S.) and subcultured when 80% confluence was reached. For patch-clamp experiments, HEK293T cells were cotransfected with plasmids containing human DIR (DRD1; GenBank accession no. NM000794), kindly provided by Dr. M. Tiberi (University of Ottawa, Ottawa, ON, Canada), the human D1R (Al-Fulaij et al., 2008), and voltage-gated calcium channel subunit CaV2.2 (Cacna2b; GenBank accession no. AF055477) with auxiliary subunits CaVβ3 (Cacnb3; GenBank accession no. M88751) and CaVα2δ1 (Cacna2d1; GenBank accession no. AF286488). Cells were cotransfected with a fixed amount of CaV2.2 and the following increasing amounts of DIR cDNA: 1, 17, and 23 ng per well (equivalent to DIR:CaV2.2 molar ratios of 0.05, 0.075, and 0.1, respectively). For some experiments, cells were additionally transfected with plasmids containing cDNA encoding a peptide corresponding to the DIR loop 1, DIR loop 2, or C terminus of CaV2.2 (0.2 µg cDNA transfected per well for saturating expression). For live imaging experiments, YFP-tagged versions of DIR and D1R were mutant used. All transient transfections were conducted using Lipofectamine 2000 (#11668019, Invitrogen; Thermo-Fisher) and, whenever necessary, an enhanced GFP (eGFP) containing plasmid to identify transfected cells and the empty plasmid pcDNA3.1 (+) to complete the total cDNA amount in the transfection mix were added. Transfected HEK293T cells were kept in culture for 24 h to allow expression, then dispersed with 0.25 mg/ml trypsin, rinsed twice, and kept in DMEM at room temperature during patch-clamp experiments.

For FRET time course of cAMP intracellular level experiments, stable HEK293T-expressing pcDNA3.1/Zeo(1)-mTurquoise2-EPAC-cp73Venus-Venus (Epac-S H187) (HEK Epac-S H187) cells were obtained by transfection of HEK293T using the K2 Transfection System (Biontex). 24 h after transfection, cells were seeded in the presence of 25 µg/ml Zeocin (InvivoGen) for 2 wk, and clonal selection was performed in 96-well plates for 2 wk. Clones were tested for Epac-S H187 by fluorescence spectra (450–650 nm) measurements in a FlexStation 3 Multi-Mode Microplate Reader (Molecular Devices) with excitation at 430 nm. The HEK Epac-S H187 clone with higher fluorescence emission was chosen for further experiments. The stable clone was grown in DMEM medium supplemented with 10% FBS, 50 µg/ml gentamicin, and 12.5 µg/ml Zeocin. For transient transfections, HEK293T and HEK Epac-S H187 cells were grown to 80–90% confluency. cDNA constructs were transfected into cells using the K2 Transfection System. The transfection protocol was optimized as recommended by the supplier. Assays were always performed 48 h after transfection. The expression of the constructs was confirmed by immunoblotting using specific antibodies. The mTurquoise2-EPAC-cp73Venus-Venus
proximal portion of the CaV2.2 C terminus was amplified by PCR and inserted into EcoRI and BamHI sites of PRK6-YFP vector. The PCR fragment was digested with EcoRI and BamHI and amplified by PCR with a sense oligonucleotide containing an EcoRI site and an antisense oligonucleotide containing a BamHI site. The PCR fragment was digested with EcoRI and BamHI and inserted into EcoRI and BamHI sites of PRK6-YFP vector. The proximal portion of the CaV2.2 C terminus was amplified by PCR with a sense oligonucleotide primer containing BamHI, an ATG codon 5207–5222, and nucleotides 5207–5222 of CaV2.2 and antisense oligonucleotide primer containing a Psyl site followed by a stop codon and nucleotides 6037–6023 of CaV2.2. The sequence for the proximal portion of the CaV2.2 C terminus was subcloned into pcDNA6 vector backbone containing IRES-eGFP. All the final constructs were Sanger-sequenced by Macrogen.

Cloning Plasmids containing cDNA encoding the DIR loop 1 (including amino acids 52–57) or DIR loop 2 (including amino acids 118–138) peptides were kindly provided by Dr. G. Zamponi (University of Calgary, Calgary, AB, Canada), originally included in pGEX-5.1 bacterial expression vectors. To express DIR loop 1 and DIR loop 2 in a mammalian system, the sequences for DIR loop 1 and DIR loop 2 were cloned by blunt ligation into a pcDNA6 vector backbone containing IRES-eGFP. The previously characterized D1RS199A mutant (Al-Fulaij et al., 2008) was generated through directed mutagenesis using the commercial plasmid containing D1RS199A mutant, the sequences without stop codon were amplified with a sense oligonucleotide containing an EcoRI site and an antisense oligonucleotide containing a BamHI site.

Drugs The commercial antipsychotic drug chlorpromazine (chlorpromazine HCl, 25 mg/ml injectable blister, CAS #50–53-3; Laboratorys Duncan S.A.) was donated by Dr. Martinez Ménaco and Dr. Pinedo (Italian Hospital of La Plata, Buenos Aires, Argentina) and used for IP injections in mice and 20-h preincubations of transfected HEK293T cells. Chlorpromazine stock solution was stored at room temperature, protected from light, and dissolved in saline solution 0.9% NaCl <24 h before IP injections. For patch-clamp recordings from mouse mPFC slices, the sodium channel blocker tetrodotoxin (1 μM; CAS #4368–28-9, #ST8024; Sigma-Aldrich), the CaV2.2 blocker ω-conotoxin-GVIA (1 μM; CAS #106375–28-4, #C-300; Alomone Labs), dopamine hydrochloride (CAS #62–31-7, #H8502; Sigma-Aldrich), (±)-1-phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol hydrochloride (SKF-38393; 10 μM; CAS #62–717–42-4, #D407; Sigma-Aldrich), and (-)-quinpirole hydrochloride (40 μM; CAS #85798–08-9, #Q102; Sigma-Aldrich) were used. Dopamine was also used in patch-clamp experiments in HEK293T cells. Cholera toxin (ChTx; 500 ng/ml; CAS #s9012–63-9, #C8052; Sigma-Aldrich) was used for 20-h preincubations in transfected HEK293T cells in order to block Gs protein activity.

Electrophysiology Calcium channel currents were recorded with either an EPC7 (HEKA Electronik) or an Axopatch 200 (Molecular Devices) amplifier. Data were sampled at 20 kHz and filtered at 10 kHz (~3 dB) using either PatchMaster (HEKA Electronik) or pCLAMP8.2 (Molecular Devices) software. Access resistance and input resistance were monitored by a step of ~10 mV. Recordings in which the access resistance increased by >20% were discarded. Leak current was subtracted online using a P/4 protocol, and recordings with leak currents over 150 pA at holding potential were discarded.

Native calcium currents of mPFC pyramidal neurons Acute coronal mouse brain slices were transferred to the recording chamber and visualized with an upright Zeiss Examiner.AI microscope (#491404–0001-000), a digital camera (Rolera Bolt Scientific CMOS; QImaging), and Micro-Manager 1.4 open source microscopy software (Vale Lab, University of California, San Francisco). Layer V/VI pyramidal neurons of the mPFC were identified by localization and morphology (Wang et al., 2006). Whole-cell patch-clamp recordings in voltage-clamp mode were conducted at room temperature (~24°C) in the previously described aCSF (2.5 mM Ca2+) under a continuous flow rate of 2.5 ml/min. Recording electrodes with resistances between 3 and 6 MΩ were used and filled with internal solution containing (in mM) 115 Cs-methanesulfonate, 20 tetraethylammonium chloride, 10 CsCl, 5 NaCl, 10 HEPES, 4 Mg-ATP, and 0.3 Na-GTP, pH 7.4, with CsOH.

For native calcium current recordings, tetrodotoxin (1 μM) was added to normal aCSF (2.5 mM Ca2+) to block native voltage-gated sodium channels. Neurons were held at resting potential (~80 mV), and native calcium currents were evoked applying square pulses from ~80 to 0 mV (60-ms duration), with a subsequent step at ~60 mV (30-ms duration) before returning to resting potential. Additionally, the specific CaV2.2 blocker ω-conotoxin-GVIA (1 μM) was added to analyze CaV2.2 contributions to total native calcium currents. Whenever indicated, 10 μM dopamine, 10 μM SKF38393, 40 μM quinpirole, or 10 μM chlorpromazine was acutely applied to the bath solution.

Calcium currents in transiently transfected HEK293T cells Whole-cell patch-clamp recordings were performed in transfected HEK293T cells in voltage-clamp mode. Recording electrodes with resistances between 2 and 5 MΩ were used and filled with internal solution containing (in mM) 134 CsCl, 10 HEPES, 10 EGTA, 4 Mg-ATP, and 1 EDTA, pH 7.4, with CsOH. All recordings were conducted at room temperature (~24°C) with an external solution containing (in mM) 140 choline chloride, 10 HEPES, 1 MgCl2·6H2O, and 2 CaCl2·H2O, pH 7.4, with CsOH. To evoke ionic CaV2.2-mediated currents in HEK293T cells, square pulses from ~100 to 10 mV (30-ms duration) followed by a step at ~60 mV (10-ms duration) were used. Cells were held at ~100 mV as resting potential. ON gating current recordings were performed with square pulses from ~100 mV to the reversal potential. The reversal potential was determined by calculating the 0 current point in a current-voltage curve built with square voltage pulses (5-ms duration) from ~100 to 55–70 mV every 0.5 mV. The datasets for each experimental condition were obtained from at least three independent experiments.
Imaging

24 h after transfection with D1R-YFP (11 or 23 ng of cDNA per well) or D1RS199A-YFP (23 ng per well), cells were washed twice with 1× PBS, and 0.5 ml of 1 µg/ml membrane marker solution (CellMask orange plasma membrane stain; Molecular Probes) was added for 1 min, then cells were washed a third time with PBS. Finally, the PBS was removed, and a clean coverslip was placed over the cell layer. Fluorescence photomicrographs were obtained using an optical epifluorescence microscope (Eclipse Nikon 50i) at 60× magnification (0.80-mm numerical aperture), equipped with B2A and G2A filters and a camera (DS-Ri1; Nikon Corp.). Image acquisition was performed using the Nis-Elements F 3.2 software (Nikon Corp.). FIJI ImageJ open source software was used to analyze the photomicrographs and to calculate total green fluorescence intensity, which was normalized by cell area. The datasets for each experimental condition were obtained from at least three independent experiments.

FRET time course of intracellular cAMP (i-cAMP)

FRET time course of intracellular cAMP (i-cAMP) was measured as previously described (Carozzo et al., 2019). Briefly, HEK293T cells transfected with D1R, D1R plus D1R loop 2, or empty pcDNA3.1 (+) plasmid was seeded in 96-well plates at a density of 10⁵ cells per well. Before each experiment was started, cells were washed with 0.9% NaCl twice, and 100 µl of FluoroBrite DMEM (Thermo-Fisher) was added to each well before placing the plate in a FlexStation 3 at 37°C. To determine i-cAMP response, the baseline fluorescence signal detected at 475 nm (donor) and 530 nm (FRET) emission with excitation at 430 nm was measured. Using the on-board pipettor, 50 µl of dopamine or chlorpromazine 3 µM stock solution (to reach a final concentration of 1 µM in the well) or FluoroBrite DMEM was added after 40 s, and then the signals were monitored every 20 s for a total of 600 s. FRET and donor intensities were measured for each time point. FRET/donor ratio was calculated and normalized to basal levels before stimulation (R/R₀) for each time point. An area under the curve value of 9-min R/R₀ i-cAMP response was calculated for each replicate.

Basal i-cAMP measurement

cAMP levels measured in HEK293T cells transfected with D1R, D1R plus D1R loop 2, or empty pcDNA3.1 (+) were performed as in Agosti et al. (2017). Briefly, 24 h after transfections, the supernatants were removed, and 0.8 ml of ethanol was added to each well. Ethanol was dried out, and residues were re-suspended with 50 mM Tris-HCl, pH 7.4, with 0.1% BSA. cAMP
content was determined by a competitive radio-binding assay for PKA using [3H]-cAMP as previously described (Davio et al., 1995). The standard curve was performed using eight cAMP concentrations ranging from 0.1 to 90 pmol. Duplicate samples in at least three independent experiments were analyzed.

Statistics

Data were analyzed and visualized using OriginPro 8 (Origin-Lab Corp.) and Prism 6 (GraphPad Software Inc.) software. We used the Kolmogorov–Smirnov test for conformity to a normal distribution, and variance homogeneity was examined using Bartlett’s (normally distributed data) and Brown–Forsythe’s (nonnormally distributed data) tests. P values were calculated from Student’s paired and unpaired t test and multiple comparisons one-way ANOVA with Dunn’s post-test versus 0 ng D1R (B). n.s., nonstatistically significant. Data were expressed as mean ± SEM, and dots represent individual data points.

Results

Chlorpromazine, a D1/D5R inverse agonist, reduces total CaV currents in mPFC neurons

First, we tested the hypothesis that D1R constitutive activity plays a role in controlling CaV2.2 current density in mPFC neurons. We obtained acute coronal brain slices and recorded native CaV currents from layer V/VI pyramidal neurons of the mPFC (Fig. 1 A). We first ran control experiments to assay the capability of D1/D5R to modulate CaV currents by comparing the effect of acute application of dopamine (10 µM) and SKF38293 (SKF; 10 µM), a specific D1/D5R agonist. We found that these two agonists equally reduced the currents (Fig. 1 B). Moreover, dopamine in the presence of 40 µM quinpirole, a D2R agonist, had a similar effect (Fig. 1 C). Thus, we conclude that D1/D5R are the main dopamine receptor subtypes modulating CaV currents in mPFC neurons. To study D1/D5R constitutive activity, we took advantage of the fact that several antipsychotic drugs, including chlorpromazine, have been shown to act not only as D2R antagonists but also as inverse agonists of D1/D5R (Cai et al., 1999). Therefore, we evaluated if pretreatment with chlorpromazine is capable of reducing CaV2.2 currents in mPFC neurons. We performed two consecutive IP injections in mice with 1 mg/kg chlorpromazine or vehicle 24 h and 1 h before the experiment and found that native CaV currents from chlorpromazine-treated mice were significantly smaller than currents from vehicle-treated mice (∼51% of the total current in control; Fig. 1 D). Next, we assessed the contribution of CaV2.2 to the total calcium current affected by chlorpromazine and found that the sensitivity of CaV currents to 1 µM ω-conotoxin GVIA was dramatically reduced from ∼18% to ∼2% in chlorpromazine-treated mice, suggesting that inhibiting D1/D5R constitutive activity reduces CaV2.2 currents (Fig. 1 E). Finally, we explored the effect of acute subsequent bath application of chlorpromazine and dopamine on CaV currents from vehicle-treated mice. We found no effect of chlorpromazine, indicating the requirement of an extended period of time for this effect, and 20% of CaV current inhibition by dopamine, similar to the values obtained in panels B.
In summary, our data suggest that pyramidal neurons from mPFC display lower CaV2.2 currents when D1/D5R constitutive activity is reduced.

D1R coexpression increases CaV2.2 currents in transfected HEK293T cells

To study the effect of D1R coexpression on isolated CaV2.2 currents, we used a heterologous expression system that we previously proved effective for studying the agonist-independent activity of other GPCRs (López Soto et al., 2015; Agosti et al., 2017). We cotransfected HEK293T cells with two different amounts of YFP-tagged D1R (D1R-YFP) cDNA and a fixed amount of CaV2.2 and its auxiliary subunits. We first confirmed that increasing the amount of transfected D1R-YFP cDNA significantly increased the total fluorescence intensity (Fig. 2A). Next, we evaluated the size of CaV2.2 currents in cells transfected with a range of D1R cDNA and found that increasing the amount of D1R cDNA dramatically increased CaV2.2 currents (Fig. 2B). Thus, we found that higher D1R expression levels led to increased CaV2.2 basal currents.

Agonist-independent activity of D1R increases CaV2.2 currents

Assuming that each D1R molecule displays a fixed level of constitutive activity, our data presented in Fig. 2 allow us to propose that D1R agonist-independent activity contributes to positively modulate CaV2.2 currents. We therefore tested if preincubation with the D1/D5R inverse agonist chlorpromazine prevents this stimulatory effect. Indeed, we found that 10 µM chlorpromazine preincubation occludes CaV2.2 current increase by D1R coexpression, while having no effect on control currents (Fig. 3). On the other hand, the drug failed to directly affect CaV2.2 currents, as we found no effect of acute application of chlorpromazine (+CPZ; 10 µM) on CaV2.2 currents in cells expressing only CaV2.2 (percent IC\textsubscript{CaV2.2} inhibition by 10 µM chlorpromazine = 2.6 ± 1.0%; n = 5; nonstatistically significant from zero, one-sample t test). We ran additional control experiments to confirm that D1R is functional in our system and the lack of effect from acute application of chlorpromazine on D1R- and CaV2.2-expressing cells. We found that dopamine application inhibits CaV2.2 currents in a concentration-dependent manner. Moreover, increasing the amount of D1R cDNA in the transfection mix had no effect on dopamine-mediated inhibition. Finally, we failed to observe an effect
of acute application of chlorpromazine in this experimental setting (Fig. 4), similar to our result on native currents (Fig. 1 F).

Together, our data from Figs. 2, 3, and 4 indicate that D1R constitutive activity is required for CaV2.2 current increase and also recapitulate our observations using chlorpromazine on native CaV currents from mouse mPFC pyramidal neurons.

Previous studies reporting D1R agonist–independent activity have used D1R mutants with diminished constitutive activity as genetic tools (Zhang et al., 2014). We chose a single mutant, D1RS199A, that displays a very low level of constitutive activity but maintains the same expression levels and sensitivity to dopamine as WT D1R (Al-Fulaij et al., 2008). We first checked that the expression level of YFP-tagged versions of WT and mutant D1R were the same (Fig. 5 A). We also compared the inhibitory effect of acute application of dopamine on CaV2.2 currents and found no differences between D1RS199A and WT D1R in our experimental conditions (Fig. 5 B). Next, we compared basal CaV2.2 currents and found that D1RS199A failed to increase basal CaV2.2 currents (Fig. 5 C). Thus, our data suggest that only constitutively active D1R is capable of increasing CaV2.2 current.

Channel-receptor physical interaction is required for D1R-induced increase of CaV2.2 currents

We next evaluated if the effect of D1R on CaV2.2 involves physical channel-receptor interaction. This hypothesis arises from published work proposing that D1R loop 2 and the proximal region of CaV2.2 C terminus interact, stimulating the traffic of CaV2.2 proteins to the cell membrane (Kisilevsky et al., 2008).

This report shows that a peptide with the sequence of D1R loop 2 competes with D1R for interaction with the channel, while other peptides, including one with the D1R loop 1 sequence, do not. Based on these observations, we assayed the effect of expressing either D1R loop 2 or 1 on the CaV2.2 current increase caused by D1R coexpression. We found that D1R loop 2 indeed occludes the current increase, while D1R loop 1 has no effect (Figs. 6, A and B). We also assayed the effect of coexpressing a 277-amino acid peptide corresponding to the CaV2.2 C terminus and found that this maneuver also prevents the current increase by D1R coexpression (Fig. 6 C). Finally, we verified that the acute inhibitory effect of dopamine in these experimental conditions was intact (Fig. 6 D). These results indicate that the CaV2.2 current increase driven by D1R requires that constitutively active D1R physically interact with CaV2.2.

D1R constitutive activity increases gating CaV2.2 currents

To explore if the increased CaV2.2 current that we observed in transfected HEK293T cells is related to a higher channel density at the plasma membrane, we recorded gating currents, a metric of the number of functional channels expressed at the cell surface. We recorded ON gating currents at the reversal potential (around +60 mV) from a resting potential of −100 mV. We calculated the charge movement as QON, normalized by the cell capacitance (Castiglioni et al., 2006). We observed that D1R coexpression increases the QON, and, consistent with our previous results, D1R loop 2 coexpression and D1R replacement by D1RS199A occluded this effect (Fig. 7 A). To demonstrate that the
QON increase is not due to changes in the relative open probability, we plotted raw QON values versus peak tail current for individual cells (Wei et al., 1994; Jones et al., 1999; Takahashi et al., 2004; Garza-Lopez et al., 2018) and found that the parameters from the lineal regression fits for control cells (−D1R) and cells coexpressing D1R (+D1R) are not different (Fig. 7 B). Taken together, our experiments allow us to propose that D1R-CaV2.2 interaction is required for the increase in CaV2.2 current that occurs when constitutively active D1R is coexpressed. Additional biochemical experiments are required to conclude that the current enhancement by D1R constitutive activity correlates with a greater number of functional CaV2.2 channels in the plasma membrane.
We explored the requirement of Gs, the main G protein reported to couple to D1R, for the effect of D1R on CaV2.2 current. We first evaluated the changes in cAMP values driven by dopamine in our experimental system. We found that acute dopamine application increases cAMP levels, reaching a maximum value in 140 s in cells expressing D1R or D1R plus the loop 2 peptide. We also confirmed that chlorpromazine failed to increase cAMP in both experimental conditions as expected for the acute application of an inverse agonist (Fig. 8 A). We next quantified the basal cAMP by radioimmunoassay and found that it has a tendency to be larger in cells expressing D1R and is significantly larger in cells coexpressing D1R and loop 2 than in control cells (Fig. 8 B). These results suggest that Gs can be acutely activated on top of its basal level of activity and that the loop 2 does not alter Gs signaling. We next assayed the Gs inhibitor ChTx (500 ng/ml) in HEK293T cells. We first corroborated that ChTx occludes dopamine-induced inhibition of CaV2.2 currents in cells coexpressing D1R and the channel (Fig. 8 C). Finally, we assessed the effect of ChTx on basal currents and found that the toxin abolishes the increase in CaV2.2 currents induced by D1R coexpression (Fig. 8 D). Taken together, our results suggest that D1R constitutive activity signals through Gs, contributing to the increase in CaV2.2 currents (Fig. 8).

Our results indicate that D1R would interact with CaV2.2, increasing the amount of channels in plasma membrane depending on its constitutive activity. On the other hand, previous work that we have replicated here in part shows that dopamine-mediated D1R activity decreases CaV2.2 currents and promotes channel internalization (Huang and Zamponi, 2017). At this point, an open question is: why do D1R constitutive and dopamine-mediated activities have opposite effects? One possibility is that D1R places more channels in the membrane in order to amplify dopamine effects when it is released from near-synaptic terminals. If this were true, we would expect that in chlorpromazine-treated mice the capability of dopamine to inhibit CaV2.2 channels would be reduced, since there would be fewer channels available. In Fig. 9, we present data supporting this thought. We recorded native currents from mPFC neurons and reproduced the result from Fig. 1 showing that in chlorpromazine-treated animals, basal native CaV currents were reduced (Fig. 9 A). More importantly, the percentage of CaV current inhibition by 10 µM SKF38393 was significantly reduced in the chlorpromazine-treated group (Fig. 9 B).

**Discussion**

Here, we demonstrated that D1R constitutive activity increases CaV2.2 currents in a heterologous expression system. Based on our experiments using an inverse agonist and a D1R mutant lacking constitutive activity, we conclude that basally active D1Rs are required to increase CaV2.2 currents. Moreover, we confirmed that D1R loop 2 and the CaV2.2 C terminus occlude this effect, suggesting a direct interaction between D1R and the channel. Surprisingly, we found that Gs activity is also required, adding a new level of complexity to the mechanism. Taking into account a previous report by the Zamponi laboratory (Kisilevsky et al., 2008), we suggest that constitutively active D1R interacts with CaV2.2, stabilizing functional channels in the plasma membrane. In this context, active Gs protein could form part of the constitutively active D1R-CaV2.2 complex, or it could be exerting an additional effect by activating a cascade that somehow modifies the channel or D1R.
Here, we propose that the chronic stimulatory effect of D1R coexpression requires Gs participation that would occur independently of Gs rapid activation by agonist binding to D1R. Our data demonstrate that basal levels of cAMP are elevated in cells expressing D1R and that acute dopamine application further increases these second messenger levels. Moreover, we found that ChTx, a specific Gs inhibitor, prevents CaV2.2 current increase by D1R coexpression as well as dopamine-induced acute CaV2.2 current inhibition. These opposite effects of D1R constitutive and dopamine-evoked activities on CaV2.2 current resemble other reports demonstrating that ORL-1 and D2R coexpression are capable of physically interacting and promoting channel trafficking toward the plasma membrane, while the agonist-mediated activation of these GPCRs acutely reduces CaV2.2 current (Huang and Zamponi, 2017). Although it is not explored in these reports, GPCRs’ constitutive activity could play a role in the basal increase of CaV2.2 trafficking. Indeed, there is evidence showing that ORL-1 (Beedle et al., 2004) and D2R (Akam and Strange, 2004; Roberts and Strange, 2005) display basal signaling. In the case of D2R, the constitutive activity is hard to evidence because D2R is coupled to Gi/o, and thus it reduces cAMP levels. On the other hand, a recent publication from our group suggests that D2R basal signaling through Gβγ impacts CaV2.2 currents (Cordisco Gonzalez et al., 2020). Thus, the dual opposite chronic and acute effects of D1R on CaV2.2 may be an example of a common modulatory behavior of some GPCRs.

We previously studied the effect of other basally active GPCRs on CaV currents with contrasting results. Ghrelin receptor constitutive activity reduces CaV2.2 forward trafficking from endoplasmic reticulum to plasma membrane and thus diminishes the CaV2.2 current in a Gi/o-dependent manner (Mustafá et al., 2017). On the other hand, melanocortin type 4 receptor reduces CaV1.2, CaV1.3, and CaV2.1 currents without affecting CaV2.2. Interestingly, melanocortin type 4 receptor is a Gs protein-coupled receptor, but its constitutive pathway acting on CaV currents is mediated by Gi/o (Agosti et al., 2014, 2017). This discrepancy reveals high heterogeneity among the effects of GPCR constitutive activity on CaV.
Here, we found that injecting mice with IP chlorpromazine, an inverse agonist of D1/D5R, dramatically reduces calcium currents in mPFC neurons. By calculating the percentage of \( \omega \)-conotoxin GVIA-sensitive current relative to the total current affected by chlorpromazine, we concluded that \( \text{CaV}_{2.2} \) accounts for \(~37\%\) of the total calcium current reduced; thus, chlorpromazine also partially decreases non-\( \omega \)-conotoxin GVIA-sensitive currents. One plausible explanation is that chlorpromazine acts directly on non-\( \text{CaV}_{2.2} \) subtypes. In this regard, an early work demonstrated that chloropromazine acutely reduces L-type calcium current in cultured mouse neuroblastoma cells (Ogata et al., 1990). On the other hand, non-\( \text{CaV}_{2.2} \) subtypes could also be sensitive to D1/D5R constitutive activity. Further experiments are required to discriminate among the possibilities of a direct effect of chlorpromazine on non-\( \text{CaV}_{2.2} \) subtypes, a contribution of D5R constitutive activity to the total effect of chlorpromazine, and the targeting of non-\( \text{CaV}_{2.2} \) subtypes by D1R constitutive activity.

The effect of D1R on \( \text{CaV}_{2.2} \) occurs quite rapidly in mouse mPFC neurons. Less than 24 h are required to observe a current reduction by chlorpromazine injection, a time range comparable to the tens of hours reported for \( \text{CaV}_{2.2} \) turnover (Bernstein and Jones, 2007; Simms and Zamponi, 2012). Moreover, it has been described that the binding of dopamine induces both D1R and \( \text{CaV}_{2.2} \) internalization (Kisilevsky et al., 2008), and another study has shown that injection of methamphetamine, an inhibitor of dopamine reuptake, reduces \( \text{CaV}_{2.2} \) currents in a D1/D5R-dependent manner (González et al., 2016). Thus, it is possible that constitutively active D1R interacts with \( \text{CaV}_{2.2} \), preventing internalization and stabilizing the channel in the plasma membrane, and that dopamine binding to D1R reverts this effect. D1R-expressing neurons would thus display a highly dynamic regulation of \( \text{CaV}_{2.2} \) expression at the cell surface.

One putative function of D1R constitutive activity is to help specify \( \text{CaV}_{2.2} \) sub-cellular localization. D1R might play a role in the noncanonical somatic and dendritic sub-localization of \( \text{CaV}_{2.2} \). The receptor might also be part of a group of modulators that localize \( \text{CaV}_{2.2} \) to postsynaptic sites, particularly in layer V/VI pyramidal neurons of the mPFC. It was recently shown that the collapsing response mediator protein 2 (CRMP2) augments \( \text{CaV}_{2.2} \) protein in PFC neurons, an effect tied to cue reinstatement after cocaine self-administration extinction (Buchta et al., 2020). Thus, it is possible that \( \text{CaV}_{2.2} \), a typically presynaptic calcium channel, is playing a noncanonical role at postsynaptic sites in PFC neurons, and that D1R, among other modulators, stabilizes \( \text{CaV}_{2.2} \) protein at these sites. Here, we showed that \( \text{CaV}_{2.2} \) currents from animals IP injected with the D1R inverse agonist chlorpromazine are less sensitive to SKF-mediated inhibition. Considering that \( \text{CaV}_{2.2} \) is highly sensitive to G protein–signaling cascades, we postulate that neurons expressing constitutively active D1R would have larger calcium currents responsive to dopamine at postsynaptic sites. Moreover, it has been reported that \( \text{CaV}_{2.2} \) localizes in dendrites in cortical pyramidal and Purkinje cerebellar neurons (Westenbroek et al., 1992) and contributes to postsynaptic calcium entry in lumbar spinal cord neurons (Heinke et al., 2004) and dentate granule cells (Hamilton et al., 2010). It would be interesting to explore the role of high-density \( \text{CaV}_{2.2} \) at PFC postsynaptic sites in terms of its contribution to activity-mediated postsynaptic calcium entry, dendrite calcium potentials, and long-term plasticity.

Antipsychotic drugs are a large heterogeneous group of D1/D5R inverse agonists that were initially described as D2R antagonists (Cai et al., 1999; Martin et al., 2001). Our work proposes that basally active D1R can be a functional target of these drugs. Here, we used chlorpromazine, a typical antipsychotic drug, and found that D1R constitutive activity plays a role in controlling \( \text{CaV}_{2.2} \) current density. To our knowledge, this is the first clear functional output of D1R constitutive activity. Besides \( \text{CaV}_{2.2} \), other synaptic proteins may also be affected by D1R constitutive activity. In particular, in hippocampal neurons, NMDA receptors complex with D1R at perisynaptic sites, and dopamine binding unleashes NMDA receptors, which migrate to the synaptic sites and contribute to long-term potentiation (Ladepeche et al., 2013). It would be interesting to study the role of D1R constitutive activity in this mechanism. Meanwhile, D5R,
the other D1-like receptor, also displays basal activity. In this regard, the inhibition of DSR constitutive activity by flupentixol, an atypical antipsychotic drug, depresses supranormal burst firing in subthalamic neurons in a rat model of Parkinson’s disease (Chetrit et al., 2013). Thus, the study of the cellular and physiological effects of DIR-like receptor constitutive activity may help to improve treatments that rely on available antipsychotics, as well as to develop more efficient drugs.

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Author contributions: All experiments presented in this work were performed in the Electrophysiology Laboratory of IMBICE. J. Raingo directed the conception and experimental design, contributed to data interpretation, and was responsible for funding acquisition. S.S. Rodríguez planned and performed the directed mutation strategy for DIRS199A, obtained the cDNA constructs for DIR-YFP, DIRS199A-YFP, and Ca_{v}2.2 C terminus, and amplified and purified the clones for the HEK293T cells transient transfections. C. Chou-Freed cloned and amplified the DIR loop 1 and DIR loop 2 clones and participated in data acquisition and analysis. C.I. McCarthy performed data acquisition, analysis, and interpretation. J. Raingo and C.I. McCarthy prepared, wrote, and revised the manuscript. A. Yaneff performed the cAMP measurement, and C. Davio guided and designed these experiments. All authors have read, critically revised, and approved the final version of this manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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