D1 receptors in the anterior cingulate cortex modulate basal mechanical sensitivity threshold and glutamatergic synaptic transmission

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Abstract

The release of dopamine (DA) into target brain areas is considered an essential event for the modulation of many physiological effects. While the anterior cingulate cortex (ACC) has been implicated in pain related behavioral processes, DA modulation of synaptic transmission within the ACC and pain related phenotypes remains unclear. Here we characterized a Crispr/Cas9 mediated somatic knockout of the D1 receptor (D1R) in all neuronal subtypes of the ACC and find reduced mechanical thresholds, without affecting locomotion and anxiety. Further, the D1R high-efficacy agonist SKF 81297 and low efficacy agonist (±)-SKF-38393 inhibit α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic receptor (AMPAR) currents in the ACC. Paradoxically, the D1R antagonists SCH-23390 and SCH 33961 when co-applied with D1R agonists produced a robust short-term synergistic depression of AMPAR currents in the ACC, demonstrating an overall inhibitory role for D1R ligands. Overall, our data indicate that absence of D1Rs in the ACC enhanced peripheral sensitivity to mechanical stimuli and D1R activation decreased glutamatergic synaptic transmission in ACC neurons.

Keywords: Dopamine, Anterior cingulate cortex, AMPA receptors, D1 receptors, D2 receptors, CFA, Inflammation, Hypersensitivity

Introduction

Dopamine (DA) binds to two classes of G protein-coupled receptor subtypes (GPCRs), classified as D1-like (D1/D5) and D2-like (D2–D4) receptors [1, 2]. A number of clinical and genetic association studies suggest that D2-like receptor mediated mechanisms have antinociceptive properties [3–6]. Yet, the role of D1-like receptors in pain modulation remains less clear. Emerging evidence has shown that ablation of spinally projecting dopaminergic neurons reduced the maintenance of long-lasting priming hyperalgesia in both sexes [7], however, spinal D5 receptors seem to play a critical role in males whereas females are more dependent on D1 receptors [8]. These studies are consistent with existing evidence supporting the pro-nociceptive effect of dopamine via a spinal D1/D5 mechanism [9]. In humans, greater functional connectivity of corticostriatal projections predict pain persistence suggesting a potential role of dopaminergic pathways in chronic pain [10]. Direct evidence from rodent studies show that dopaminergic projections from the ventral tegmental area (VTA) to the medial prefrontal cortex (mPFC) triggers antinociceptive signals [11] and optogenetic phasic activation of VTA-mPFC...
projections reduces mechanical hypersensitivity during neuropathic states [12].

The anterior cingulate cortex (ACC) is one of the primary cortical regions involved in the affective component of pain [13]. Human imaging studies have shown excitation of ACC neurons with noxious stimuli [14] and animals models suggest that the activity of ACC glutamatergic synapses is integral for encoding chronic pain [15, 16] and anxiety [17]. In the ACC, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic receptors (AMPA Rs) mediated excitatory postsynaptic currents (EPSCs) are enhanced in brain slices from animal models of chronic pain [17–21]. The ACC expresses both D1- and D2-like receptors [22, 23], with transcripts of both receptors showing a decrease following nerve injury [24]. Further, a single microinjection of DA or amantadine, a NMDA receptor antagonist into the ACC reduced autotomy behavior following nerve injury, while D1 or D2 antagonists injected into the ACC blocked the antinociceptive effects of amantadine [25]. It has also been shown that the ACC potentiates spinal cord transmission by forming direct facilitating glutamatergic synapses with spinal dorsal horn neurons (SDH), while optogenetic activation of ACC-SDH projecting neurons increased mechanical sensitivity [26].

In cortical regions such as the mPFC, D1R signaling has been shown to increase AMPAR expression in neuronal cultures [27, 28] and promote long-term potentiation of glutamatergic synapses [28]. Alternatively, D2R signaling downregulates AMPAR levels in mPFC neuronal cultures [27] and dephosphorylates AMPAR subunits necessary for induction of synaptic potentiation [28]. Whole-cell patch clamp recordings from mPFC principal neurons have demonstrated that D1Rs enhance AMPAR EPSCs [29], while D2Rs inhibit AMPAR EPSCs in the ACC [30]. Based on pharmacological studies using brain slices from the ACC, DA has been shown to inhibit AMPAR EPSCs through a D2R signaling mechanism; an effect that is enhanced by co-application of a D1R antagonist [30]. Hence, we wanted to directly determine the contribution of D1-like receptors in modulation of synaptic activity within the ACC. Furthermore, since the primary dopaminergic system connects the VTA with the limbic system and D1Rs may modulate affective sensory inputs [31], we decided to determine the contribution of D1Rs in the ACC to a pain related behavioral phenotype.

To this end, we used CRISPR/Cas9 mediated somatic gene knockout (sKO) to investigate the functional role of D1Rs in the adult ACC. Behavioral testing revealed decreased mechanical sensitivity thresholds in mice with sKO for D1Rs, while locomotion and anxiety phenotypes remained unchanged. By using whole-cell patch clamping on brain slices from mice, we observed an inhibitory role for D1-like agonists on AMPAR mediated currents, mimicking the effects of DA on EPSCs [30]. The inhibitory effect of D1 agonists was not blocked by D1 antagonists, but rather co-application of both ligands resulted in a synergistic and robust inhibition of AMPAR currents, suggesting potential novel effects for these drugs.

Materials and methods

Animals

Rosa26-LSL-Flag-Cas9 knockin mice (or floxCas9-EGFP mice) (Stock No: 024857, The Jackson Laboratory) [32] were used for viral injections, immunohistochemistry and behavior. Mice were maintained on a 12-h light/dark cycle with ad libitum access to food and water. Male adult (6 to 8 weeks of age) C57BL/6 J mice were acquired from the Jackson Laboratory (Bar Harbor, ME) and used for electrophysiological characterization of D1R drugs. All mice were housed in groups of 4 upon arrival and procedures were conducted in accordance with the animal care standards set forth by the Canadian Council on Animal Care (CCAC) and were approved by the University of Toronto Animal Care Committee. All animals were maintained within a temperature-controlled environment (20 ± 1 °C) with a 12:12 h light:dark cycle. A compressed cotton nesting square and crinkled paper bedding were provided in each cage as a source of environmental enrichment. All mice had access to food (Harlan Teklad 8604) and water ad libitum.

DNA constructs

Strategies used for CRISPR editing gene knockout (KO) have been described previously [33]. To knockout the Drd1 gene, 20-nt target sequence (Drd1 sgRNA) in the unique exon of the gene was selected using an online CRISPR design tool (http://crispr.mit.edu/) to minimize off-target activity. For in vitro validation of the Drd1 KO, the guide oligonucleotide was cloned into px459 vector (Addgene plasmid # 62988). To generate sgRNA expressing AAV viral vector (pAAV Drd1-sgRNA-mCherry) for in vivo use, the guide was cloned into pX552 (Addgene plasmid # 60958) vector by single step cloning using SapI restriction sites.

Cell line culture, transfection, and TIDE analysis

Neuro-2A (N2A) cells were grown in high glucose DMEM containing 10% FBS, penicillin/streptomycin and L-glutamine (HyClone-GE Healthcare, Logan, UT). Cells were maintained at 37 °C in 5% CO2 atmosphere and transfected using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer’s protocols. Confluent (50–70%) N2A cells were transfected with a px459 based construct (pX459 vectors with Drd1-sgRNA). To select only transfected cells, 48 h after transfection cells were incubated with 3 μM
puromycin for 72 h followed by 48 h incubation without puromycin. To isolate genomic DNA for TIDE analysis, cells were lysed by tail buffer (Tris pH = 8.0 0.1 M, NaCl 0.2 M, EDTA 5 mM, SDS 0.4% and proteinase K 0.2 mg/ml), and DNA was precipitated using isopropanol followed by centrifugation (13,000 g 15 min). DNA was resuspended in TE Buffer (10 mM Tris pH 8.0, 0.1 mM EDTA) and used for PCR to amplify guide site with: Forward primer sequence GAGGGACTTCTCTTTTCCG CAT and Reverse primer sequence CCAGCAGCAGACGAATACCC. PCR products were sent to sequencing with Forward primers and frequencies of mutations were determined by online TIDE tool (https://tide.nki.nl/).

**AAV viral particle preparation**

For pAAV Drd1-sgRNA/mCherry, AAV serotype 5 viral particles were produced by the University of North Carolina (UNC) Vector core facility. AAV5-hSyn-mCherry and AAV5-hSyn-Cre were purchased from UNC Vector core facility (Chapel Hill, NC, US).

**Mouse stereotaxic surgery**

Mice were anesthetized with a preparation of ketamine 10 mg/ml and xylazine 1 mg/ml (0.1 ml/10 g, i.p.). The animal was placed in a stereotaxic frame and the skull surface was exposed. Two holes were drilled at injection sites and 300 nl of a mixture of AAV-hSyn-Cre and AAV-hSyn-mCherry (control) or AAV-Drd1-sgRNA-mCherry was injected into the ACC (injection coordinates: AP: + 1.0; ML: ±0.3; DV: −1.35) or into STR (injection coordinates: AP: + 1.1; ML: ±1.2; DV: −3.7) using an injector with microsyringe pump controller (WPI) at the speed of 4 nl per second.

**Immunohistochemistry**

After behavioral tests, mice were rapidly anesthetized with Avertin injection (2.5% tribromoethanol, 0.2 ml/10 g, i.p.) and transcardially perfused with 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (pH 7.5). Brains were post-fixed overnight in the same solution and stored in 4 °C. Forty microgram thick sections were cut using a vibratome (Leica, Microsystems, ON, Canada) and stored in PBS at 4 °C. Sections were mounted using fluorescence mounting medium (Dako Omnis, Agilent). Images were obtained using the EVOS M7000 Imaging System (Thermo Fisher Scientific, Massachusetts).

**Synaptosome isolation and Western blot**

Synaptosomes were isolated using Syn-PER reagent according to manufacturer’s recommendation (Thermo Fisher Scientific). Briefly, dissected and frozen brain tissue was lysed in Syn-PER solution. Samples were centrifuged for 10 min at 1200 g. After discarding the pellet, samples were centrifuged for another 20 min at 15000 g to obtain synaptosomes in the pellet. The pellet was resuspended in Syn-PER solution. Protein concentration was measured by using a DC-protein assay (Bio-Rad, Hercules, CA, US). Protein extracts were separated on precast 4–20% Tris-glycine gels (Thermo Fisher Scientific, Waltham, MA) and transferred to nitrocellulose membranes. Blots were immunostained overnight at 4 °C with primary antibodies mouse anti-GAPDH (1:5000, Santa Cruz sc-322,333) and rat anti-Drd1 (1:500, Sigma d2940) Blots were incubated with secondary antibodies for 1 h at room temperature with goat anti-mouse IR Dye 680 (1:10000, Mandel 926–68,020) and goat anti-rabbit IR Dye 800 (1:10000, Mandel 926–32,211). Immune complexes were revealed using iBright Western blot imaging systems (Thermo Fisher Scientific, Waltham, MA). Quantitative analyses of fluorescent IR dye signal were carried out using Image Studio Lite 5.2 software. For quantification, GAPDH was used as a loading control for the evaluation of total protein levels.

**Behavioral tests**

**Open field test (OFT)**

The OFT was performed for 30 min in an automated Omnitech Digiscan apparatus (AccuScan Instrument, Columbus, OH). Each mouse was placed in a corner of a large Plexiglas box and the total distance and the time spent in center (25% of the total surface) were recorded separately.

**Dark-light emergence test (DLET)**

The DLET was performed for 5 min with mice placed initially at the center of the dark chamber. Tests were conducted using an automated open field activity apparatus with light/dark insert (Med-Associates, St Albans, VE) with the light compartment illuminated at 800 lx. The total time spent in the light compartments was used for analysis.

**Elevated plus maze (EPM)**

EPM was performed for 5 min with mice initially placed in the center of the maze. Mice were video tracked using Viewer software (Biobserve behavior research). The time spent in the open arm was measured.

**Y-maze test**

The test was performed in a Y-maze made of opaque plastic arms. This test is based on the innate curiosity of mice to explore novel areas and presents no negative or positive reinforcers and very little stress for the mice [34]. Briefly, mice were placed into one of the arms of the maze (arm “A”) and allowed to explore the maze with one of the arms closed (new arm) for 10 min. After an hour intertrial interval, mice were returned for 5 min to the Y-maze (with all arms open) and placed into
the same start arm (test trial). Mice were video tracked using Viewer software (Biobservé behavior research). The total distance travelled during the test trial and the number of visits into each arm were measured.

**Von Frey test**
Mice were placed on an elevated metal mesh floor within small Plexiglas cubicles (9 × 5 × 5 cm high) and allowed to habituate for 1 h before testing began. An automated von Frey test was used (Ugo Basile Dynamic Plantar Aesthesiometer) to measure withdrawal responses. In this assay, pressure is gradually increased by the device until the mouse withdraws its hind paw; the maximal pressure at that point is displayed. Measurements were taken in both hind paws.

**CFA model of inflammatory pain**
Complete Freund’s adjuvant (CFA; Sigma Aldrich, CA) was injected subcutaneously in a volume of 20 μl into both plantar hind paws using a 100-μl Hamilton syringe with a 30-gauge needle. Both paws were injected to induce bilateral activation of ACC. The automatic von Frey test was used to measure hypersensitivity of the hind paws to mechanical stimuli.

**Tissue preparation for electrophysiology**
Brain slice preparations were done as previously described [30, 35]. Mice were anesthetized with 5% isoflurane and killed by decapitation. The brains were quickly removed and placed in cold (4 °C) oxygenated (95% O₂; 5% CO₂) artificial cerebrospinal fluid (ACSF) consisting of 124 mM NaCl, 4.4 mM KCl, 2 mM CaCl₂, 1 mM MgSO₄, 25 mM NaHCO₃, 1 mM NaH₂PO₄, and 10 mM glucose. Brain slices (300 μm) containing coronal sections of the ACC were prepared with a VT1200S tissue slicer (Leica, Concord, ON). Slices recovered for a minimum of 60 min in a submerged holding chamber (25 °C) before recording.

**Whole-cell patch-clamp recording**
Slices were removed from the holding chamber and placed in a recording chamber where they were continuously perfused with oxygenated (95% O₂; 5% CO₂) ACSF at a rate of 2 ml per min. Whole-cell voltage-clamp recordings from superficial layers of pyramidal neurons of the ACC region were obtained under visual guidance using a 40X objective on a Zeiss Axioskop FS upright microscope. Recordings were made with electrodes (4–6 MΩ) fabricated using a horizontal puller (P1000; Sutter, Novato, CA) and filled with an internal solution containing, 145 mM K-glucuronate, 5 mM NaCl, 1 mM MgCl₂, 0.2 mM EGTA, 10 mM HEPES, 2 mM Mg-ATP, and 0.1 mM Na₃-GTP (adjusted to pH 7.2 with KOH). Neurons were voltage-clamped at −60 mV using an Axon 700B amplifier (Axon Instruments, Foster City, CA), low-pass filtered at 1 kHz, and digitized at 10 kHz with Climplex (version 10.6; Molecular Devices). Evoked EPSCs (eEPSCs) were stimulated by a tungsten bipolar stimulation electrode placed (Microprobes, Gaithersburg, MD) on the slice surface at the deep layers of the ACC proximal to the patched neuron. AMPA- and kainate-mediated eEPSCs were isolated by adding picrotoxin (100 μM) to the ACSF to block GABAₐ (γ-aminobutyric acid type A)-receptor–mediated inhibitory synaptic currents (Additional file 1: Supplemental Figure1). For paired pulse facilitation recordings, paired stimulation (50 ms apart) was performed every 30 s. Stable baseline recordings were obtained for 5 min followed by the perfusion of pharmacological agents. Input resistance and access resistance were monitored continuously throughout each experiment; experiments were terminated if these changed by > 15%. Only recordings with stable holding current and series resistance maintained below 25 MΩ were considered for analysis.

**Pharmacological agents**
The drugs used in the experiments include SKF 81297, (±) SKF 38393, R (+)-SCH-23390 hydrochloride, SCH 39166, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and picrotoxin. All drugs were purchased from Sigma Aldrich, CA or Tocris.

**Data and statistical analysis**
Data were collected and analyzed using pClamp 10.6 software (Molecular Devices, San Jose, Ca). Data in five-minute bins were analyzed with sample traces corresponding to the averaged traces from these five-minute bins. For experiments where a washout phase after drug application was measured, one- or two-way analysis of variance (ANOVA) was used to compare the EPSCs during course of drug action versus the baseline and the washout phase. Tukey’s HSD was used for post-hoc analysis where appropriate. We used t-test comparisons to determine whether baseline and drug effects were significantly different. *p < 0.05 was considered statistically significant.

**Results**
Development of a CRISPR/Cas9 mediated somatic Drd1 knockout strategy
We used the CRISPR/Cas9 gene editing technology to inactivate Drd1 expression in ACC neurons of adult mice. This approach involves the use of conditional floxed-Cas9 mice [36]. Two AAVs respectively encoding a Cre recombinase or a sgRNA targeting the gene of interest are used in combination to activate Cas9 and induce a somatic gene knockout (sKO) [33]. This approach avoids developmental compensation mechanisms...
and allows for brain region specific manipulation of gene expression [36].

First, a guide RNA (gRNA) selective for a unique exon of the D1R gene (Drd1 sgRNA) was designed (Fig. 1a) and efficacy of the single guide RNA was measured by Tracking of indels by decomposition (TIDE) analysis in N2A cells (Fig. 1b). Multiple deletions and insertions were detected with one nucleotide insertion accounting for 77% of mutated DNA sequences (position “+1”). Initially, to validate the in vivo efficacy of the CRISPR/Cas9 protocol, we generated sKO of Drd1 in the striatum, a brain region known to highly express Drd1 [1]. A mixture of AAV Syn-Cre with AAV Drd1-SgRNA-mCherry (Drd1-SgRNA) or AAV Syn-mCherry (control) was microinjected into the striatum of floxCas9-EGFP mice (Fig. 1c). Three weeks following delivery of AAV constructs, D1R expression was significantly reduced in synaptosomal preparations from Drd1-SgRNA mice, relative to control mice (Fig. 1d). Hence, in vitro and in vivo delivery of CRISPR/Cas9 components efficiently knocked down the Drd1 gene to generate brain region specific sKO for the D1R in adult mouse.

ACC specific DRD1 somatic knockout modulates pain related behavior in adult mice

To investigate the functional role of D1Rs in the ACC, we delivered AAV Syn-Cre + AAV Drd1-SgRNA-mCherry (ACC_D1R-KO) or AAV Syn-mCherry (control mice) bilaterally into the ACC of floxCas9-EGFP mice and performed behavioral tests 3 weeks post injection (Fig. 2a). Bilateral injections were performed to avoid potential hemispheric modulation of behavior. mCherry fluorescence of AAV Drd1-SgRNA-mCherry was detected in cell bodies in ACC and fibers from ACC projecting neurons were present in STR confirming the specificity of the injection site into ACC (Fig. 2b). To assess whether disruption of D1R expression in the ACC changed behavior, we first measured the total distance travelled in an open field test as proxy for locomotion. No significant differences were observed between the ACC_D1R-KO and control mice over 30 min of testing (Fig. 2c). However, the time spent in the center of the open field by ACC_D1R-KO mice was significantly higher than control mice (Fig. 2d). Behavioral assessment using the elevated plus maze (EPM) as a measure of anxiety, revealed no significant difference between control and ACC_D1R-KO mice (Fig. 2e). Furthermore, in the dark-light emergence test (DLET) as an additional measure of anxiety, we found no significant difference between control and ACC_D1R-KO mice (Fig. 2f). Based on our results, ACC D1Rs do not seem to be involved in the modulation of anxiety. We also assessed working memory by using the Y-maze test [34], but found no significant difference between ACC_D1R-KO and control mice (Fig. 2g).

As a measure of pain, we assessed mechanical thresholds using automatic von Frey testing because enhanced mechanical sensitivity is consistently associated with

![Fig. 1 Specificity of CRISPR/Cas9 mediated somatic knockout of Drd1 in Striatum. a Drd1 targeting sequence and corresponding protospacer adjacent motif (PAM). b Evaluation of Drd1 targeting sgRNA by Tracking of indels by decomposition (TIDE) analysis in Neuro2A cells. c Schematic diagram of experimental design. d Expression of Drd1 in synaptosomes from control (Fold change D1R/Gapdh: 100 ± 17.57% relative to control; n = 5 mice) and Drd1-sgRNA KO striatum mice (Fold change D1R/Gapdh: 37 ± 9.03% relative to control; n = 5 mice) (t-test; t8 = 3.20, *p < 0.05)
**Fig. 2** (See legend on next page.)

**A**

AAV syn-Cre + AAV Drd1-SgRNA/mCherry or AAV syn-mCherry (control) in ACC

floxCas9-EGFP mouse → Behavior

Day 0 → Day 21

**B**

Images showing fluorescence in ACC

**C**

Graph showing total distance travelled (cm)

- Control
- ACC_D1-KO

**D**

Graph showing time in the center (s)

- Control
- ACC_D1-KO

**E**

Graph showing time spent in open arm (s)

- Control
- ACC_D1-KO

**F**

Graph showing time in the light zone (s)

- Control
- ACC_D1-KO

**G**

Diagram showing 1 h → New arm

**H**

Graph showing % of visits and total distance travelled

- Control
- ACC_D1-KO

Graph showing naive threshold (g)

- Basal
- 4d CFA

Legend on next page.
ACC hyperactivity [17, 20, 26, 37]. In ACC_D1R-KO mice, paw withdrawal thresholds were significantly decreased compared to control mice indicating that loss of D1Rs in the ACC may increase mechanical sensitivity (Fig. 2h). Since ACC synapses are selectively potentiated in the complete Freund’s Adjuvant (CFA) model of inflammatory pain [17, 38], and D1Rs have been shown to be involved in synaptic potentiation mechanisms after challenging mice with the CFA model of inflammatory pain. Four days following CFA, mechanical thresholds decreased in both ACC_D1R-KO and control mice compared to baseline responses; however, thresholds were no longer significantly different between genotypes (Fig. 2h).

D1-like receptor agonists inhibit eEPSCs in the ACC

Previous electrophysiology studies characterizing D1R modulation of basal AMPAR mediated transmission have yielded mixed results that seem to depend on species, brain region, neuronal subtype, concentration of drug and type of event recorded (see Table 1). However, since we observed that knocking down D1Rs in the ACC decreased mechanical thresholds, we postulated that D1Rs may have an inhibitory effect on ACC basal excitatory transmission.

To test for this, we performed whole cell patch recording of neurons in the ACC layers II-III and V and recorded eEPSCs. After obtaining stable baseline transmission for 5 min, a high efficacy D1R agonist SKF 81297 (30 μM) [1, 53], was perfused for 10 min and subsequently washed out. The application of SKF 81297 (30 μM) significantly reduced the amplitude of eEPSCs, which returned to baseline following washout (SKF 81297 30 μM, 75.87% ± 2.75%; washout: 97.17% ± 2.7%, Fig. 3a and CI). Furthermore, a lower concentration of SKF 81297 at (10 μM) inhibited eEPSC amplitudes, which also returned to baseline following washout (SKF 81297 10 μM: 80.46% ± 6.27%; washout: 96.37% ± 5.47%, Fig. 3b and CI). The percentage of inhibition mediated by SKF 81297 30 and 10 μM is shown in Fig. 3d for comparison.
presynaptic plasticity and a measure of presynaptic function [55]. A single stimulus was delivered, followed 50 ms later by a second stimulus. Baseline eEPSCs were recorded using the paired pulse stimulation protocol and SKF 81297 (30 μM) was applied after 5 min of baseline recording. Inhibition mediated by SKF 81297 (30 μM) did not significantly change PPR relative to the baseline (97.02 ± 5.42% of baseline; Fig. 3f). Unaltered PPR and blockade of inhibition by SKF 81297 (30 μM) with GDP-beta-S (2 mM) in the pipette indicates a postsynaptic mechanism of action by SKF 81297. Also, the inhibitory effect of (±)-SKF-38393 (50 μM) had no significant effect on PPR of eEPSCs (104.42% ± 7.45% of baseline, Additional file 1, Supplemental Figure 3D). Hence, both D1R ligands mediate inhibition of eEPSCs by postsynaptic mechanisms.

**D1R selective antagonists, SCH-23390 and SCH 39166 fail to block inhibition by D1 agonists**

Since our data showed that D1-like agonists inhibit eEPSCs, we reasoned that administration of a D1-like antagonist would block these effects. For these experiments, we used the well-studied pharmacological agent SCH-23390, a known inhibitor of D1-like receptors [28, 56, 57]. We recorded baseline eEPSCs and applied SCH-23390 followed by application of SKF 81297. In previous work, a high concentration of DA antagonists was required to block DA modulation of eEPSCs in the ACC [30]; therefore, we administered a higher concentration of SCH-23390 (60 μM) than SKF 81297 (30 μM). To our surprise, co-application of SCH-23390 and SKF 81297 produced a robust and significant inhibition of eEPSCs, which returned to baseline levels following washout (SCH-23390 + SKF 81297: 28 ± 6.46%; washout: 87.69 ± 6.46%; Fig. 4a and c). Similarly, SCH-23390 (60 μM) produced similar inhibition of eEPSCs when co-applied with (±)-SKF 38393 (50 μM) (35.67% ± 4.75% of baseline) and washout of drugs returned eEPSC amplitude towards baseline levels (81.77% ± 11.45% of baseline, Additional File 1, Supplemental Figure 3A and B).

Since the inhibition of eEPSCs by SKF 81297 was enhanced in the presence of a high dose of SCH-23390 (60 μM), we presumed that SCH-23390 may have off-target effects at high concentrations. Hence, we used SCH-23390 at a lower concentration (10 μM), which has been shown to block dopaminergic effects in PFC brain slice preparations [53, 56]. Pre-application of SCH-23390 (10 μM) failed to block inhibition of eEPSCs followed by application of SKF 81297. In previous work, a high concentration of DA antagonists was required to block DA modulation of eEPSCs in the ACC [30]; therefore, we administered a higher concentration of SCH-23390 (60 μM) than SKF 81297 (30 μM). To our surprise, co-application of SCH-23390 and SKF 81297 produced a robust and significant inhibition of eEPSCs, which returned to baseline levels following washout (SCH-23390 + SKF 81297: 28 ± 6.46%; washout: 87.69 ± 6.46%; Fig. 4a and c). Similarly, SCH-23390 (60 μM) produced similar inhibition of eEPSCs when co-applied with (±)-SKF 38393 (50 μM) (35.67% ± 4.75% of baseline) and washout of drugs returned eEPSC amplitude towards baseline levels (81.77% ± 11.45% of baseline, Additional File 1, Supplemental Figure 3A and B).

**Table 1** Electrophysiology studies characterizing D1R modulation of basal AMPAR mediated transmission. D1R agonist modulation of basal AMPAR mediated currents

| D1R agonist | Species | AMPAR current | Result | Neuron Type | Synaptic locus | Region | Reference |
|-------------|---------|---------------|--------|-------------|----------------|--------|-----------|
| SKF 81297 (30 μM) | Rat | eEPSC | Inhibit | Magnocellular | Presynaptic | BF | [39] |
| DA (30 μM) | Rat | eEPSC, mEPSC | // | // | // | // | |
| SKF 81297 (30 μM) | Rat | eEPSC | Inhibit | Cholinergic | Presynaptic | BF | [40] |
| SKF 81297 (1 μM) | Rat | eEPSC | Inhibit | Pyramidal layer V | Presynaptic | PFC | [41] |
| SKF 38393 (10 μM) | Rat | eEPSC | Enhance | Pyramidal Layer II/III | Postsynaptic | PFC | [29] |
| DA (250 nm-5 μM) | Monkey | eEPSC | Inhibit | Pyramidal layer III | Undetermined | PFC | [42] |
| SKF 81297 (1 μM) | Rat | eEPSC | Enhance | Pyramidal Layer II/III | Postsynaptic | PFC | [43] |
| SKF 81297 (5 μM) | Mice | eEPSC | No effect | Pyramidal | – | PFC | [28] |
| DA (50 μM) | Mice | sEPSC | Enhance | D1R+ neurons | Presynaptic | Dorsal Striatium | [44] |
| SKF 81297 (10 μM) | // | // | // | // | // | // |
| SKF 81297 (1 μM) | Rat | Kainite EPSC | Cultured MSN | Striatum | | | [45] |
| SKF 38393 (10 μM) | Rat | AMPA EPSC | Enhance | MSN | Postsynaptic | Dorsal Striatium | [46] |
| SKF 38393 (5 μM) | Rat | eEPSC | Enhance | MSN | – | Striatum | [47] |
| // | // | glutamate EPSC | No effect | // | // | – |
| DA (75 μM) | Rat | eEPSP | Inhibit | Field & whole cell | Presynaptic | NAc | [48] |
| SKF 81297 (30 μM) | // | // | No effect | LTD | // | // |
| SKF 38393 (100 μM) | // | // | // | // | // | // |
| DA (50 μM) | Mice | eEPSC | Inhibit | MSN | Postsynaptic | NAc | [49] |
| SKF 38393 (10 μM) | // | // | // | // | // | // |
| SKF 81297 (5 μM) | Mice | Kainate EPSC | Enhance | MSN | Postsynaptic | Neostriatum | [50] |
| SKF 81297 (5 μM) | Chicken | mEPSC | No effect | Pyramidal layer II/III | Motoneurons | – | ACC | [51] |
| SKF 38393 (10 μM) | Mice | glutamate EPSC | Enhance | – | Embryonic | | [52] |

Studies were selected based on activity mediated by D1R pharmacological agonists and antagonist, BF Basal forebrain, PFC Prefrontal cortex, NAc Nucleus Accumbens, ACC Anterior Cingulat Cortex, eEPSC Stimulation evoked excitatory postsynaptic current, mEPSC miniature excitatory postsynaptic potential, Kainate EPSC and AMPA EPSC EPSC Evoked by application of kainite or AMPA, LTD Long-term Depression, MSN Medium spiny neuron
Fig. 3 (See legend on next page.)
amplitude by a low concentration of SKF 81297 (10 μM) (69% ± 8.49% of baseline; Fig. 4b and c). Similarly, inhibition of eEPSCs by (±)-SKF 38393 (50 μM) and a high concentration SKF 81297 (30 μM) were not blocked by SCH-23390 (10 μM) compared with baseline transmission and washout phase (one-way ANOVA, F_{2,32} = 31.22, p < 0.001). II: eEPSCs were significantly reduced by SKF 81297 10 μM compared with baseline transmission and washout phase (one-way ANOVA, F_{2,32} = 4.27, p = 0.02). d No difference in the inhibition of AMPAR mediated eEPSCs by SKF 81297 30 μM and 10 μM, (t-test, t_{13} = 0.62, p = 0.506). e I: Averaged sample traces and averaged and normalized data (n = 4/11 mice). II: SKF81297 30 μM did not produce significant change in baseline transmission in presence of GDP-beta-S 2 mM in pipette (paired t-test, t_{13} = 0.43, p = 0.6831). f I: Averaged sample traces and averaged and normalized data (n = 8/6 mice). II: No significant difference in PPR following SKF 81297 30 μM application when compared with baseline responses (paired t-test, t_{13} = 0.52, p = 0.62). *p < 0.05, **p < 0.01

Discussion

Excitation of cortical brain regions is consistently observed in animal models of chronic pain [15], and enhanced AMPAR mediated eEPSCs responses are one of the established mechanisms for this observed effect [17–20, 26, 37, 59]. In the current paper, we combined emerging evidence for involvement of mesolimbic dopaminergic system in modulation of pain [60–62] with the question of how the dopaminergic system modulates AMPAR transmission in the ACC. This led us to investigate the role of an ACC dopaminergic receptor subtype in modulation of behavior and AMPAR transmission. Indirect evidence suggests that D1Rs are involved in the modulation of synaptic transmission in the ACC [30]. Hence, we wanted to specifically define a role for D1R signaling in the ACC in the context of pain. By using CRISPR/Cas9 methods, we generated a sKO for D1Rs in ACC neurons of adult mice. This method is particularly useful as it avoids developmental compensation mechanisms [36, 63]. Behavioral testing of ACC_D1R-sKO mice, revealed that baseline mechanical thresholds were significantly lower in comparison to control mice, but without any alteration in locomotion, anxiety, working memory, and hypersensitivity in the CFA model of inflammatory pain. These results indicate a selective role for ACC D1Rs in modulating baseline sensory responses in pain naïve mice.

Since enhanced glutamatergic transmission in the ACC is correlated with decreased mechanical sensitivity [17, 18], decreased mechanical thresholds in ACC_D1R-KO mice suggests that D1Rs may have an inhibitory role on glutamatergic transmission in the ACC. Consistent with this idea, low and high concentrations of the D1R agonist SKF 81297 and (±)-SKF 38393 reduced AMPA R eEPSC in ACC slices. These results are similar in nature to the mechanism of exogenously applied DA in ACC slices from a previous report [30]. However, D1Rs are Gα_s/olf-coupled and stimulate adenylyl cyclase and cAMP production, while D2Rs are Gα_i-coupled and inhibit adenylyl cyclase and cAMP production [64]. This typically leads to D1Rs and D2Rs serving excitatory and inhibitory modulation of neuronal excitability, respectively [53, 65]. In the striatum and mPFC, D1Rs enhance AMPAR EPSCs through upregulation of PKA activity [29, 45, 47, 52, 66]. This upregulation is mediated by an upregulation of GluR1 AMPARs [27] and phosphorylation at Ser^{845} [28, 45], a mechanism necessary for induction of long-term potentiation in the ACC [17, 59]. D1Rs also inhibit AMPAR EPSCs in the mPFC [41] and coactivation of D1- and D2Rs inhibit EPSCs in the primate mPFC, an effect that is blocked by the D1R antagonist SCH-23390 [42]. Likewise, in the ACC, PKA activity upregulates synaptic AMPAR transmission through insertion of calcium permeable AMPARs and phosphorylation of GluR1 at Ser^{845} [17, 21, 59, 67]. Further, a traditionally Gα_s-coupled receptor, such as the D1R is also capable of neuronal inhibition through N-
Fig. 4 (See legend on next page.)
type Ca$^{2+}$ channel modulation indicating that D1Rs may display promiscuity in their G$\alpha$ subunit coupling [68]. In neostriatal neurons, activation of D1Rs inhibit N-type calcium channel activity via a PKA-dependent pathway [69], while new data suggest that D1R inhibition of neurons in the ACC may occur through modulation of hyperpolarization-activated cyclic nucleotide–gated (HCN) channels [70].

An inhibitory role for D1Rs is contradictory to the pro-excitatory properties of D1R signaling as shown in previous work on PFC neurons [28, 29]. Studies on ACC synaptic plasticity in chronic pain have exclusively focused on long-term potentiation as the mechanism for encoding long-lasting pain [15, 16, 59]. Based on our results, we observe an inhibitory effect by D1R agonists on basal transmission and the reduction of basal sensory thresholds in ACC_D1R-KO mice, making it probable that D1Rs exert inhibition of basal AMPAR transmission. In support of this, a recent study has shown that ACC neurons form direct synapses with SDH to modulate mechanical sensitivity through glutamatergic synapses [26]. Hence, modulation of ACC descending facilitatory projections by D1Rs is a possible mechanism of action. Further research is required to explore the modulation of ACC-SDH projecting neurons by D1Rs.

In our experiments and previous work, D1R agonists and dopamine inhibit AMPAR EPSC transmission by an average of 20–25% [30]. Thus, we speculate that the moderate change in pain sensitivity observed in the ACC_D1R-KO mice is physiologically appropriate and expected rather than a larger effect. However, it is important to appreciate that the pain phenotype we have observed is novel and unique. This is the first time that the D1 receptor is specifically downregulated in the ACC of adult mice. In addition, this is the first time a DA receptor subtype in a cortical region has been shown to be a modulator of basal peripheral sensitivity to mechanical stimuli. Thus far, studies on ACC glutamatergic transmission and pain have focused on plasticity mechanisms in response to injury [15, 17–19, 28], leaving basal synaptic transmission without a well-defined functional role. The phenotype of ACC_D1R-KO mice warrants further research, especially toward exploring dopamine receptor signaling in the sensory and affective modulation of pain.

Our current experiments used a sgRNA targeting Drd1 as a “knockout condition” where gene expression was knocked down (e.g. 80% reduction of gene expression) in affected cells, however this approach also generates a controlled mosaic of genome disruption. So, if the expression of the gene is knocked out in affected cells, the gene structure may be perfectly normal in adjacent cells. This approach is not a knockout or knock-in in the classical sense of what would be achieved using a germinal manipulation, but represents a somatic knockout and intersectional (a more neural circuit selective method) knockout as previously described by us [36, 63, 71]. Although the ACC has a well-defined role in anxiety, ACC_D1R-KO mice do not seem to have a robust change in anxiety behavior, except for more time spent in the center of the open field. This prompted us to test these mice on two other tests of anxiety (EPM and DLET), which did not reveal any further differences. In the published literature there is a poor correlation between performance in the OFT and EPM test, while EPM and DLET exhibit a greater correlation [72]. In addition, OFT correlates well with freezing responses in contextual fear conditioning, while EPM does not suggesting that the OFT may be a more useful metric of locomotor activity [73].

Despite our results with D1R agonists and their similarity in function to DA [30], we cannot conclusively claim that inhibition by SKF 81297 and (±)-SKF 38393 are mediated exclusively by D1Rs. The inhibitory effects of SKF 81297 and (±)-SKF 38393 were not blocked by SCH 23390 and SCh 39166, two widely used D1R antagonists. Co-application of D1-like agonists (i.e. high concentration of either SKF 81297 or (±)-SKF-38393) in combination with a high concentration of SCH 23390 (a D1-like receptor antagonist)
antagonist) produced a robust synergistic depression of AMPAR currents in ACC slices, similar to a synergistic depression induced by SCH 23390 and DA in a previous report [30]. Since, SCH 23390 also acts as an agonist for 5HT2c receptors [74–76], which inhibit AMPAR eEPSCs in the ACC [77], one possibility for the observed synergistic depression of AMPA currents is non-specific binding of SCH 23390 to serotonin receptor subtypes. These results further emphasize the need for pharmacological studies on genetically defined D1R+ neurons [53]. However, it is important to note that the D1R antagonist, SCH 39166 is clinically effective in treating tics related to pediatric [78] and adult [79] Tourette’s syndrome. In addition to pain, excitation of the ACC has also been associated with Tourette’s syndrome [80]. Hence inhibition of ACC AMPAR currents may be a contributing mechanism for the clinical effects of SCH 39166.

Despite the paradoxical pharmacology, there are reasons to believe that inhibition of AMPAR eEPSCs is mediated by D1Rs. First, both SKF 81297 and (±)-SKF 38393 at two different doses have the same effect on eEPSCs and it would be unlikely for two separate agonists at different doses to have the same effect by independent off-target activity. Second, the inhibitory effect of SKF 81297 and (±)-SKF 38393 mimic the effects of dopamine from ACC brain slices in terms of degree of inhibition, synaptic locus of activity and reversibility of the inhibition [30]. Lastly, inhibition of basal transmission by D1R correlates with the observed mechanical sensitivity phenotype in the ACC_ D1R-KO mice. These data provide the first evidence that D1Rs in the ACC modulate peripheral sensitivity to mechanical stimuli based on somatic genetic manipulations. In addition, correlational pharmacological data for D1Rs have revealed a potential novel inhibitory mechanism for D1Rs in the cingulate cortex on basal glutamatergic transmission. Further research is imperative to demonstrate the precise mechanism of D1R modulation of mechanical sensitivity, and the functional role of ACC basal transmission in behavior.

Supplementary information
Supplementary information accompanies this paper at https://doi.org/10.1186/s13041-020-00661-x.

Additional file 1 Figure S1. AMPAR mediated evoked EPSCs in the anterior cingulate cortex. Figure S2. AMPAR mediated eEPSC modulation by the D1R agonist (±)-SKF-38393. Figure S3. D1R antagonists do not block D1R agonist effects in ACC slices.

Abbreviations
ACC: Anterior Cingulate Cortex; ACSF: Artificial cerebrospinal fluid; AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic receptors; CFA: Complete Freund’s adjuvant; D1R: D1 receptor; D2R: D2 receptor; DLET: Dark-light emergence test; EPSCs: Excitatory postsynaptic currents; EPM: Elevated plus maze; GPCR: G protein-coupled receptor subtypes; gRNA: Guide RNA; LTD: Long-term depression; OFT: Open field test; mPFC: Medial prefrontal cortex; SDH: Spinal dorsal horn; sKO: Somatic gene knockout; VTA: Ventral tegmental area

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Authors’ contributions
SDG performed electrophysiological and behavioral experiments. CQ performed CRISPR/Cas9 and behavioral experiments. SDG, CQ and LJM analyzed the data. SDG and LJM wrote the manuscript. JMB and LJM designed/supervised the overall project. The authors reviewed, edited and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
All procedures were conducted in accordance with the animal care standards set forth by the Canadian Council on Animal Care (CCAC) and were approved by the University of Toronto Animal Care Committee.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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