Gi-DREADD Expression in Peripheral Nerves Produces Ligand-Dependent Analgesia, as well as Ligand-Independent Functional Changes in Sensory Neurons

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Designer receptors exclusively activated by designer drugs (DREADDs) are an advanced experimental tool that could potentially provide a novel approach to pain management. In particular, expression of an inhibitory (Gi-coupled) DREADD in nociceptors might enable ligand-dependent analgesia. To test this possibility, TRPV1-cre mice were used to restrict expression of Gi-DREADDs to predominantly C-fibers. Whereas baseline heat thresholds in both male and female mice expressing Gi-DREADD were normal, 1 mg/kg clozapine-N-oxide (CNO) produced a significant 3 h increase in heat threshold that returned to baseline by 5 h after injection. Consistent with these behavioral results, CNO decreased action potential firing in isolated sensory neurons from Gi-DREADD mice. Unexpectedly, however, the expression of Gi-DREADD in sensory neurons caused significant changes in voltage-gated Ca²⁺ and Na⁺ currents in the absence of CNO, as well as an increase in Na⁺ channel (NaV1.7) expression. Furthermore, CNO-independent excitatory and inhibitory second-messenger signaling was also altered in these mice, which was associated with a decrease in the analgesic effect of endogenous inhibitory G-protein-coupled receptor activation. These results highlight the potential of this exciting technology, but also its limitations, and that it is essential to identify the underlying mechanisms for any observed behavioral phenotypes.

Key words: analgesia; DREADD; GPCR; nociception; pain; second messenger

Introduction
Thirty percent of the adult population suffers from chronic pain because none of the currently available therapies are consistently effective or devoid of deleterious side effects (Portenoy et al., 2007; Moore et al., 2010). Thus, there is a critical need for alternative approaches for the treatment of pain. Nociceptive afferent hyperexcitability is thought to underlie most chronic pain conditions, making this neuron an obvious therapeutic target. An ideal way to treat pain would therefore be to inhibit nociceptive afferents via the selective expression of an inhibitory receptor that can be activated by an exogenous compound and has no effect on endogenous binding sites or receptors. Designer receptors activated by designer drugs (DREADDs) provide a methodology to test this exciting possibility.

The Gi-DREADD, also known as human muscarinic receptor 4 (hM4Di), is a mutated muscarinic acetylcholine receptor that, when activated by its ligand, clozapine-N-oxide (CNO), initiates the inhibitory G-protein (Gi) signaling cascade that inhibits neuronal signaling and/or excitability. Canonically, the Gi signaling cascade is inhibitory via two major second-messenger mechanisms: (1) the Giα subunit-mediated inhibition of adenyl cyclase; (2) the coupling of Gi to pertussis toxin-sensitive G-protein-coupled receptors, leading to the inhibition of adenyl cyclase activity.

Received Sept. 17, 2015; revised Aug. 2, 2016; accepted Aug. 23, 2016.

Author contributions: J.L.S., N.N.S., L.M.S., S.E.R., B.M.D., and M.S.G. designed research; J.L.S., N.N.S., L.M.S., B.M.D., and M.S.G. performed research; J.L.S., N.N.S., B.M.D., and M.S.G. contributed unpublished reagents/analytic tools; J.L.S., N.N.S., L.M.S., S.E.R., B.M.D., and M.S.G. analyzed data; J.L.S., N.N.S., S.E.R., B.M.D., and M.S.G. wrote the paper.

This work was supported by NIH Grants NS073548 (J.L.S., L.M.S.), NS086749 (J.L.S.), NS084661 (N.N.S.), NS083347 (M.S.G.), CA177857 (B.M.D.), AR063772 (S.E.R.), and AR064445 (S.E.R.).

The authors declare no competing financial interests.

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class (AC) activity (Sadana and Dessauer, 2009) that is involved in regulation of transcription factors as well as numerous protein kinase A (PKA)-regulated processes that maintain/increase neuronal excitability and (2) the direct binding of Giβγ subunits to activate G-protein inwardly rectifying potassium channels (e.g., GIRKs and Kv3 channels) as well as to inhibit voltage-gated calcium channels (VGCCs; Herlitze et al., 1996; Ikeda, 1996; Ruiz-Velasco and Ikeda, 2000).

In the CNS, Gi-DREADD has been used to inhibit neuronal firing (Wess et al., 2013; Lee et al., 2014), disrupt memory consolidation when Gi-DREADD expression is targeted to ventral hippocampal glutamatergic neurons (Zhu et al., 2014), and drive schizophrenia-like behavior when targeted to parvalbumin or GAD65 interneurons (Nguyen et al., 2014). These results suggested that targeted expression of Gi-DREADD is not only feasible but enables the selective inhibition of the targeted neurons in the CNS. Recent results support the possibility that Gi-DREADDs could likewise be used to inhibit neurons in the PNS (Iyer et al., 2016). Thus, the goals of this study were to further explore the possibility that Gi-DREADDs are a viable tool for manipulating sensory afferents to modulate nociception and pain, as well as determine the mechanism(s) responsible for these actions.

Using behavioral, histological, and molecular biological assays and electrophysiological recordings, we show that Gi-DREADD activation in sensory neurons decreases excitability, thereby producing antinociception or analgesia. However, expression of Gi-DREADD under regulation of TRPV1-cre produces significant changes in voltage-gated Ca2+ and Na+ currents and disruption of both excitatory and inhibitory second-messenger signaling in the absence of CNO. Although Gi-DREADD expression in peripheral nerves does decrease excitability, unexpected ligand-independent activity may discourage use of this tool as a modulator of disease conditions, including persistent pain.

Materials and Methods

Animals

For conditional expression of Gi-DREADD in nociceptors, TRPV1-cre mice (http://jaxmice.jax.org/strain/017769.html) were crossed with mice harboring the RC:PD1 allele (Ray et al., 2011), in which expression of Gi-DREADD from the Rosa locus is cre dependent. Throughout the remainder of this study, we refer to these mice as $V^{iG}$-DREADD mice. Gi-DREADD is a modified version of the hM4Di. Both male and female mice were used for all experiments. Controls were littermates lacking the TRPV1-cre allele. Animals were housed in the Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility at the University of Pittsburgh in a 12 h light/dark cycle with a temperature- and Accreditation of Laboratory Animal Care-accredited facility at the University of Pittsburgh in a 12 h light/dark cycle with a temperature- and Accreditation of Laboratory Animal Care-accredited facility at the University of Pittsburgh in a 12 h light/dark cycle with a temperature- and Accreditation of Laboratory Animal Care-accredited facility at the University of Pittsburgh in a 12 h light/dark cycle with a temperature- and Accreditation of Laboratory Animal Care-accredited facility at the University of Pittsburgh in a 12 h light/dark cycle with a temperature-

Behavior

Heat. Behavioral measures were made using a planar testing apparatus (IITC) in the University of Pittsburgh Rodent Behavior Analysis Core. Mice (control, $n = 20$; Gi-DREADD, $n = 19$) were acclimatized for 1 h in the thermal testing apparatus before testing. Animals were placed individually in a small Plexiglas enclosure with a glass floor. A focused light beam was applied to the plantar surface of the hindpaw. The mean of three measures of paw withdrawal latencies (PWLS) was determined at baseline and 1, 3, and 5 h after CNO injection (1 mg/kg, i.p.; Sigma). A separate group of mice (control, $n = 25$; Gi-DREADD, $n = 25$) received an intraplantar injection of DAMGO or vehicle (5 μg/paw) in a volume of 5 μl, followed by intraplantar prostaglandin E2 (PGE2; 100 ng/paw) in a volume of 10 μl (Levine and Taiwo, 1989; Taiwo and Levine, 1990; Yang and Gereau, 2002). PWL was determined at baseline and 45 min after PGE2.

Mechanical. Hindpaw withdrawal frequencies in response to von Frey filament stimulation were performed as described previously (Wang et al., 2013). Mice (control, $n = 10$; Gi-DREADD, $n = 10$) were acclimatized for 1 h before testing. Mechanical stimuli were applied from below to the plantar surface of the right hindpaw (~2–3 s), and responses to the applied monofilament (VF #3.61, equivalent to 0.4 g of force) were determined. The stimulus was applied 10 times at baseline and 1 h after CNO injection (1 mg/kg, i.p.; Sigma). The number of positive responses was converted into a percentage, with 10 positive responses corresponding to 100%. The experimenter was blinded to mouse genotype for all behavioral tests.

Primary DRG culture

Mice were anesthetized with isoflurane and transcardially perfused with cold Ca2+/Mg2+-free HBSS (Invitrogen). Bilateral DRG (L4–L6) were dissected into cold HBSS and dissociated as described previously (Malin et al., 2010). Cells were cultured in DMEM/F-12 (Invitrogen) containing 10% fetal bovine serum (FBS) and antibiotics (penicillin/streptomycin, 50 U/ml). Two hours later, coverslips were flooded with Leibovitz’s L-15 medium (Invitrogen) containing 10% FBS, 5 mM HEPES, and 5 mM glucose and stored at room temperature. Experiments were performed within 8 h of tissue harvest.

Patch-clamp physiology

At least three mice, including males and females, were used for all studies. Within the text, $n$ refers to the number of neurons.

Current clamp. Whole-cell patch–clamp recording were used to assess changes in the excitability of cultured DRG neurons. Borosilicate glass electrodes were filled with (in mM) 110 K-methanesulfonate, 30 KCl, 5 NaCl, 1 CaCl2, 2 MgCl2, 10 HEPES, 11 EGTA, 2 Mg-ATP, and 1 Li-GTP, pH 7.2 (adjusted with Tris-base), 310 mOsm (adjusted with sucrose). Neurons were continuously superfused with a bath solution that contained (in mM) 3 KCl, 130 NaCl, 2.5 CaCl2, 10 HEPES, 11 EGTA, 2 Mg-ATP, and 1 Li-GTP, pH 7.4 (adjusted with Tris-base), 325 mOsm (adjusted with sucrose). CNO-induced changes in accommodation [action potential (AP) generation] were assessed by a ramp-and-hold protocol consisting of a 250 ms ramp followed by 500 ms of sustained current injection. Accommodation was determined by counting the number of APs evoked during this protocol. The magnitude of current injection was adjusted so as to evoke an AP during the ramp phase of the stimulation protocol. This protocol was then used to stimulate neurons every 15 s before and after application of 10 μM CNO, where at least three stimuli were used to establish the stability of neuronal excitability before the application of CNO. CNO was applied for 3 min in the majority of experiments. To facilitate comparisons between neurons, CNO-induced changes were analyzed as a percentage change from baseline.

To begin to identify mechanisms underlying the actions of CNO, passive and active electrophysiological properties were assessed. Passive properties assessed included resting membrane potential ($V_m$), capacitance, and input resistance ($R_m$, $R_i$). $R_i$ was estimated with the aid of amplifier circuitry before and 90–180 s after the application of CNO. Active
electrophysiological properties were assessed with an AP evoked by a 4 ms depolarizing current pulse. These included the following: AP duration at 0 mV, magnitude of AP overshoot, magnitude of the afterhyperpolarization (AHP), and AHP decay (τ AHP). The magnitude of the overshoot was measured from 0 mV. The magnitude of the AHP was measured from the Vm. Decay of the AHP was estimated by fitting the decay phase of the AHP with a single exponential function.

Voltage clamp. In a subpopulation of neurons studied in current clamp, voltage-clamp data were collected before and after application of CNO to assess the properties of the current responsible for the inhibitory actions of CNO. Currents were evoked twice from each neuron before and after CNO application with the same voltage-clamp protocol: once from a holding potential of −60 mV and a second time from a holding potential of −80 mV. The protocol consisted of 100 ms, 5 mV steps from −80 to −40 mV. To detect the presence of an increase in “leak” current, a p/4 leak subtraction protocol was not used. Series resistance compensation (>70%) was used for all voltage-clamp recordings.

To isolate Ca²⁺ and Na⁺ currents, electrodes (1–4 MΩ) were filled with an electrode solution containing (in mM) 100 Cs-methanesulfonate, 5 Na-methanesulfonate, 40 TEA-Cl, 2 MgCl₂, 11 EGTA, 10 HEPES, 2 Mg-ATP, and 1 Li-GTP, pH 7.2 (adjusted with Tris-base), 310 mOsM (adjusted with sucrose). The bath solution for Ca²⁺ was isolated (in mM) 100 choline-Cl, 30 TEA-Cl, 2.5 CaCl₂, 0.6 MgCl₂, 10 HEPES, and 10 glucose, whereas the solution for Na⁺ current isolation contained (in mM) 35 NaCl, 65 choline-Cl, 30 TEA-Cl, 0.1 CaCl₂, 5 MgCl₂, 10 HEPES, and 10 glucose. Both bath solutions were pH 7.4 (adjusted with Tris-base), and osmolality was adjusted to 325 mOsm with sucrose. Voltage-gated Na⁺ currents (VGSCs) were evoked from a holding potential of −60 mV. To isolate the slowly inactivating tetrodotoxin (TTX)-resistant Na⁺ current (TTX-R Iₗₛ) from the more rapidly activating and inactivating TTX-sensitive Na⁺ currents (TTX-S Iₗₛ), a steady-state inactivation protocol was used to determine the conditioning step amplitude needed for full availability of TTX-S Iₗₛ, as well as the potential at which TTX-S Iₗₛ was completely inactivated. These two potentials were then used as 500 ms conditioning steps before the collection of current–voltage (I–V) data, which consisted of 15 ms voltage steps from −50 to +50 mV in 5 mV increments (with a 5 s interstep interval). TTX-S Iₗₛ was separated from the total current by digitally subtracting the TTX-R Iₗₛ from the total current. Na⁺ current conductance–voltage (G–V) data were estimated from I–V data based on the measured reversal potential. Voltage-gated Ca²⁺ currents were evoked from a holding potential of −60 mV. I–V data were generated with 50 ms voltage steps from −70 to +45 mV in 5 mV steps. Ca²⁺ current conductance–voltage (G–V) data were measured directly from the instantaneous I–V associated with tail currents evoked during the collection of I–V data. To minimize the impact of leak currents on isolation of voltage-gated Na⁺ and Ca²⁺ currents, a p/4 protocol was used for Na⁺ currents, with a holding potential of −70 mV during the acquisition of the leak pulses, whereas a p/4 protocol was used for Ca²⁺ currents with a holding potential of −90 mV during the acquisition of the leak pulses. G–V data were fitted with a Boltzmann equation to estimate the maximal conductance (Gmax) and the potential at which half-maximal conductance was activated (V1/2). To monitor drug effects and stability of evoked current, peak inward currents were then used as 500 ms conditioning steps before the collection of G–V data. TTX-S was completely inactivated. These two potentials, a p/4 leak subtraction protocol was not used. Series resistance compensation (>70%) was used for all voltage-clamp recordings.

Drugs

CNO (Sigma) was dissolved in DMSO to a stock concentration (100 mM) and then further diluted in sterile saline (1 mg/kg, behavior) or recording buffer (10 μM, electrophysiology). DAMGO (Tocris) was dissolved in water to a stock concentration of 1 mM and then further diluted in recording buffer (1 mM, electrophysiology) or sterile saline (1 μg/μl, 5 μl, i.pl., behavior). PGE₂ (Sigma) was dissolved to a stock concentration of 10 mg/ml in ethanol and then further diluted to a working concentration of 10 nM in sterile saline. Forskolin (10 and 30 μM; Tocris) was dissolved to a stock concentration of 20 mM in DMSO and then further diluted to the working concentration in recording buffer.

**Table 1. PCR primers**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession number</th>
<th>Primer</th>
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<tbody>
<tr>
<td>KCNJA1 (GIRK1, Kir1.1)</td>
<td>D45022 (PrimerBank)</td>
<td>Forward: GGGGACCATATCCAGCAGTTG</td>
</tr>
<tr>
<td>KCNJA1 (GIRK1, Kir1.2)</td>
<td>NC_000082.6</td>
<td>Reverse: CCGTACGTCCTCTCTCTG</td>
</tr>
<tr>
<td>KCNJA1 (GIRK3, Kir3.3)</td>
<td>U11860 (PrimerBank)</td>
<td>Forward: AAGGACCTCGTCCCTGTAACG</td>
</tr>
<tr>
<td>KCNJA1 (GIRK4, Kir3.4)</td>
<td>U33631 (PrimerBank)</td>
<td>Reverse: CGGCTGATCTTACGGAATGCT</td>
</tr>
<tr>
<td>SCN9A (Nav1.7)</td>
<td>NM_018852.2</td>
<td>Reverse: TTGCCACATACGTGGTGGTAC</td>
</tr>
<tr>
<td>SCN10A (Nav1.8)</td>
<td>NM_009134.2</td>
<td>Reverse: TTGGACACATGCGCTCTATCC</td>
</tr>
<tr>
<td>CACNA1A (Cav2.1)</td>
<td>NC_000074.6</td>
<td>Reverse: ATCGGACTAAGCTGCGTTC</td>
</tr>
<tr>
<td>CACNA1B (Cav2.2)</td>
<td>NC_000068.7</td>
<td>Reverse: GTACGATCAAGGATGCTTTC</td>
</tr>
<tr>
<td>Oprm1 (μ opioid receptor)</td>
<td>NC_000076.6</td>
<td>Reverse: GCCCTCAACTTTATGTGTTGTA</td>
</tr>
<tr>
<td>Oprd1 (δ opioid receptor)</td>
<td>NC_000070.6</td>
<td>Reverse: GTCTCCATACGTGGTACACCT</td>
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<tr>
<td>Oprk1 (κ opioid receptor)</td>
<td>NC_000067.6</td>
<td>Reverse: GTGGGCTTATGGGCAAT</td>
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<tr>
<td>CHRM4 (hmACHR4)</td>
<td>NM_000741.3</td>
<td>Reverse: GACAGACTCCTGAGGACCA</td>
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<tr>
<td>GAPDH</td>
<td>NC_000072.6</td>
<td>Reverse: ATGGTCTCCTGATGGATGCTGA</td>
</tr>
</tbody>
</table>

| PrimerBank | Reverse: ATGGGACGTTGCGTTCCTTCT  |

Shown is a list of primers used to assess effects of Gi-DREADD expression on endogenous GPCR transcription.

**qPCR**

DRG RNA was isolated from seven to nine mice per group using the RNeasy mini kit (Qiagen). RNA (0.5 μg) was treated with DNase (Invitrogen) to remove genomic DNA and reverse-transcribed using Superscript II reverse transcriptase (Invitrogen). Real-time PCR was used as described previously to determine the extent of expression of various channels and receptors in DREADD-expressing sensory neurons compared with control littermates (Malin et al., 2006). Primers were designed using Oligo software (Molecular Biology Insights) and the Harvard PrimerBank (Wang et al., 2012). Primer sequences used are listed in Table 1. SYBR Green PCR amplification was performed using a Bio-Rad CFX connect real-time system. After amplification, a dissociation curve was plotted against the melting temperature to ensure amplification of a single product. All samples were run in duplicate, and as a negative control, reactions were run with the reverse transcribed control reaction products but without template. The relative fluorescence of SYBR Green bound to double-stranded DNA was compared with a passive reference for each cycle. Threshold cycle (Ct) values, the cycle number in which SYBR Green fluorescence rises above background, were recorded as a measure of initial template concentration. Relative fold changes in RNA levels were calculated by the ΔΔCt method using GAPDH as a reference standard: Ct values from duplicate samples were averaged and subtracted from the reference standard, yielding ΔCt. The difference between the ΔCt of target genes from experimental and control groups were then calculated (ΔΔCt). Relative expression was determined as 2 ΔΔCt.

**Statistical analysis**

Student’s t test was used to compare two groups, whereas statistical analyses for differences over time in multiple groups were performed on GraphPad Prism software using two-way repeated-measures ANOVA followed by the Holm–Sidak post hoc test. Data from males and females were pooled unless, as where indicated, significant sex differences were detected. Data are presented as the mean ± SEM. For all analyses, significance was set at p < 0.05.

**Results**

Peripheral expression of Gi-DREADDs

To investigate the utility of Gi-DREADDs for the inhibition of nociception, Gi-DREADDs were expressed in C-fibers using the
TRPV1-cre allele (see Materials and Methods) combined with a cre-responsive Gi-DREADD effector allele (Ray et al., 2011). We subsequently refer to these mice as V1Gi-DREADD mice. Because the expression of TRPV1 is broader during embryonic development than in the adult (Cavanaugh et al., 2011), TRPV1-cre results in recombination in the majority of C-fibers. To confirm successful recombination and Gi-DREADD expression, we used an anti-HA-488-conjugated antibody to locate Gi-DREADD-expressing cells in the DRG and spinal cord (Fig. 1A). No cell bodies were labeled within the spinal cord, but a band of immunoreactivity in lamina I/II of the dorsal horn was observed, consistent with Gi-DREADD expression in primary afferent terminals (Fig. 1A). In L4 DRG, 67 ± 1.7% of neurons expressed Gi-DREADD compared with the total absence of HA immunoreactivity in control littersmates (Fig. 1B). Greater than 87% of Gi-DREADD-expressing neurons are IB4 or CGRP positive, 39 ± 0.9% and 47 ± 1.8%, respectively (Fig. 1C). Moreover, 84 ± 2.1% of IB4-positive neurons and 80 ± 2.0% of CGRP-positive neurons express Gi-DREADD, all with small to medium-sized (≤450 μm²) somata. Furthermore, HA immunoreactivity was present in predominantly small neurons (Fig. 1D), suggesting that TRPV1-cre was successful in restricting expression of Gi-DREADD to C-fibers and a small population of putative A-δ nociceptors.

Activation of Gi-DREADDs induces heat analgesia but has no effect on mechanical sensitivity
To determine the effect of activated inhibitory DREADDs on heat thresholds, V1Gi-DREADD mice and control littermates were tested using the Hargreaves’ assay for heat sensitivity. Baseline PWL was similar in both V1Gi-DREADD mice and control mice (Fig. 2A). However, 1 h after CNO injection (1 mg/kg, i.p.), V1Gi-DREADD mice exhibited a significant increase in PWL lasting for 3 h (Fig. 2B; F(3,51) = 5.537, n = 9–10 per group, **p < 0.01. Ctrl, Control. The mice tested for changes in mechanical sensitivity were tested for thermal sensitivity on two different occasions to confirm the efficacy and activity of CNO. Similar to the cohort shown in Figure 2A, these V1Gi-DREADD mice also exhibited a

Figure 1. HA-tagged Gi-DREADD expression in L4 DRG. A, Gi-DREADD expression was confirmed by staining with an HA-488-conjugated antibody. Scale bars, 100 μm. B, The majority (67 ± 1.7%) of neurons in L4 DRG express the HA-tag for Gi-DREADD (solid green bar, HA +). C, Gi-DREADD is expressed in predominantly IB4- and CGRP-positive neurons. D, Gi-DREADD is expressed in predominantly small (150–300 μm²) neurons. n = 4.

Figure 2. The effect of CNO activation on thermal and mechanical sensitivity. A, Raw data showing no significant difference in baseline thermal sensitivity between control (black) and V1Gi-DREADD (green) mice. B, CNO (1 mg/kg, i.p.) significantly increased thermal thresholds for 3 h after injection. C, CNO (1 mg/kg, i.p.) had no detectable influence on mechanical sensitivity. Changes were assessed with a two-way repeated-measures ANOVA; n = 9–10 per group, **p < 0.01. Ctrl, Control.
significant increase in PWL to radiant heat \( (F_{(1,12)} = 14.10, n = 10 \) per group, \( p < 0.01 \)). These data demonstrate that a systemic dose of 1 mg/kg CNO produces heat, but not mechanical analgesia, and that CNO can be given multiple times and still produce a comparable degree of analgesia.

CNO-Gi-DREADD signaling reduces neuronal excitability and modulates AP shape

To identify the mechanism(s) underlying the actions of CNO in the \( \text{V}^{\text{Gi-DREADD}} \) mice, whole-cell current-clamp experiments were performed on dissociated DRG (L4–L6) neurons from \( \text{V}^{\text{Gi-DREADD}} \) and littermate control mice to measure the effect of CNO on primary afferent excitability. In neurons from \( \text{V}^{\text{Gi-DREADD}} \) mice, application of CNO (10 \( \mu \)M) resulted in a significant decrease in accommodation (Fig. 3A,B), a hyperpolarization of the membrane potential (Fig. 3C), and a decrease in input resistance (\( R_{\text{gi}} \); Fig. 3D). There was no significant difference between neurons from male and female mice with respect to the proportion of neurons in which CNO reduced excitability, nor was there a difference in the magnitude of the decrease in excitability (data not shown; Fisher’s exact test, \( p = 0.09 \)).

The active electrophysiological parameters, as defined in Figure 4A, were compared between neurons from control and \( \text{V}^{\text{Gi-DREADD}} \) mice (Table 2). CNO had no effect on any parameter measured in neurons from littermate controls but induced a significant decrease in accommodation after 10 \( \mu \)M CNO (striped) in neurons from \( \text{V}^{\text{Gi-DREADD}} \) mice (green, \( n = 22 \)) compared with littermate controls (black, \( n = 6 \)). CNO also resulted in a significant decrease in membrane potential (C) and input resistance (D) in the same populations plotted as a percentage change from baseline. Statistical analysis was assessed for all measures using a two-way ANOVA with Holm–Sidak post hoc analysis where \( * p < 0.05, ** p < 0.01 \).

Gi-DREADD expression suppresses AC signaling and voltage-gated \( \text{Na}^+ \) currents in the absence of CNO

The smaller AP overshoot in DRG neurons from \( \text{V}^{\text{Gi-DREADD}} \) mice in the absence of CNO suggested that there was a constitutive suppression of VGSCs in these neurons. To directly assess this possibility, we recorded VGSCs in isolated sensory neurons from \( \text{V}^{\text{Gi-DREADD}} \) and control mice in voltage clamp (Fig. 5A). First, we focused on the TTX-R \( I_{\text{Na}} \) because it has been shown to be the dominant, if not the only, VGSC to underlie the upstroke of the action potential in putative nociceptive DRG neurons (England et al., 1996). Although there was no difference between groups with respect to half-maximal voltage (\( V_{1/2} \); Table 3; unpaired Student’s \( t \) test, \( p = 0.129 \)), maximal conductance (\( G_{\text{max}} \)) was significantly smaller in neurons from \( \text{V}^{\text{Gi-DREADD}} \) mice (11.5 ± 2.2 pS/pF, \( n = 15 \)) compared with neurons from control littermates (20.2 ± 3.9 pS/pF, \( n = 10 \); Fig. 5B; unpaired Student’s
Potassium current changes underlying CNO-induced inhibition. A, A representative AP from a neuron from a V1Gi-DREADD mouse in which we defined the active electrophysiological properties analyzed. t is the time constant of decay. B, Left, GIRK1 and GIRK2, but not GIRK3 and GIRK4, mRNA are expressed in mouse DRG. Right, Pooled data show that there is no significant difference in GIRK1/2 expression between DRG from V1Gi-DREADD mice (green, n = 7–9 per group) and littermate controls (Ctrl; black, n = 7–9 per group); mRNA expression changes were assessed with a two-way ANOVA. SC, Spinal cord. C, A population of neurons demonstrating suppression in spike number in response to CNO (green, n = 6) also displayed a significant increase in voltage-gated K⁺ currents (VGKCs) with step depolarization to −45 and −40 mV in response to CNO application. D, In neurons in which there was no effect of CNO in current clamp (n = 9), no change in VGKCs was detected. There was no difference in the baseline current density between CNO responders (1.53 ± 0.2 pA/pF) and nonresponders (1.47 ± 0.2 pA/pF) at 50 mV. However, after CNO, the differences are significant at −50, −45, and −40 mV (two-way repeated-measures ANOVA, *p < 0.05).

Table 2. CNO-induced changes in active electrophysiological properties in neurons from V1Gi-DREADD mice (n = 15) and littermate controls (n = 10)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>V1Gi-DREADD</th>
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<tbody>
<tr>
<td></td>
<td>Before CNO</td>
<td>After CNO</td>
</tr>
<tr>
<td>AP overshoot (mV)</td>
<td>51.4 ± 1.3</td>
<td>51.3 ± 0.6</td>
</tr>
<tr>
<td>Duration (ms)</td>
<td>4.7 ± 0.3</td>
<td>4.8 ± 0.3</td>
</tr>
<tr>
<td>AHP (mV)</td>
<td>−12.9 ± 2.4</td>
<td>−13.9 ± 1.7</td>
</tr>
<tr>
<td>AHP decay (t, ms)</td>
<td>241.1 ± 73.4</td>
<td>174.2 ± 55.1</td>
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</table>

* A single 4 ms depolarizing current injection was used to measure CNO-induced changes in active properties as defined in Fig. 4B. Two-way ANOVA with Holm–Sidak post hoc analysis assessed active properties. **p < 0.05, main effect of genotype; *p < 0.05, interaction between genotype and CNO treatment; **p < 0.01, interaction between genotype and CNO treatment. *p < 0.01, post hoc comparison of CNO treatment within genotype.

modulated by G-protein-coupled receptor (GPCR) signaling (Maruyama et al., 2004; Rush and Waxman, 2004; Ostman et al., 2008; Vanoye et al., 2013), the activation of this current is so slow that it makes little direct contribution to the upstroke of the action potential (and would not have been detectable in the 15 ms voltage steps used to study Na⁺ currents in these neurons). Additionally, whereas a CNO-induced decrease in a persistent Na⁺ current could have contributed to the membrane potential hyperpolarization as well as the decrease in excitability observed, such a change should have been associated with an increase in membrane resistance. Because a decrease in membrane resistance was observed, the possibility of changes in Nav1.9-mediated currents was not pursued further.

Although originally included as a control for potential changes in Nav1.8 expression, we detected a significant influence of genotype on Nav1.7 expression in DRG, with V1Gi-DREADD mice expressing significantly more Nav1.7 (Fig. 5C; Student’s t test, p = 0.031; n = 7–8 per group). Nav1.7 is the TTX-sensitive α subunit thought to underlie the majority of TTX-S INa in putative nociceptive neurons (Zhang et al., 2013). To determine whether this increase in expression was associated with an increase in TTX-S INa, a voltage-clamp protocol was used to isolate TTX-S currents for direct study (Gold et al., 1996a). Consistent with the PCR data, the TTX-S current density (Fig. 5D) was significantly larger in neurons from V1Gi-DREADD mice (−26.7 ± 6.0

Figure 5. Nav1.8 channel expression did not appear to contribute to a change in channel expression in the V1Gi-DREADD mice as there was no significant influence of Nav1.8 mRNA levels (Fig. 5C). Analysis of TTX-R INa by sex and genotype (two-way ANOVA) revealed a significant influence of genotype (lower peak current density and Gmax in neurons from V1Gi-DREADD compared with controls) and sex (lower peak current density and Gmax in females compared with males) but no significant interaction. Although Nav1.9 has also been implicated in the control of nociceptive afferent excitability and shown to be
Gold et al., 1998
McCleskey, 2000
Kawano et al., 2009

Inhibition of VGCCs appears to be a common mechanism of inhibitory GPCR signaling in CNS neurons and there is evidence that $K_{IR}$ currents are present in nociceptive afferents (Gao et al., 2007; Kawano et al., 2009), there is little direct evidence of Gi–$K_{IR}$ signaling in rodent DRG neurons (McCleskey, 2000). Instead, inhibition of VGCCs (McCleskey, 2000) and AC (Taiwo and Levine, 1991) appears to be the primary mechanisms responsible for the actions of inhibitory GPCR activation. Therefore, evidence of CNO-induced activation of a $K^+$ current in our current and voltage-clamp experiments was unexpected and raised the possibility that Gi-DREADD expression may have altered normal Gi signaling in these neurons. To test this possibility, we first sought to determine changes in $G_{max}$ of $V_{1Gi-DREADD}$ neurons normalized to the average $G_{max}$ of controls.

**Table 3. Biophysical properties of TTX-R and TTX-S Na$^+$ currents in neurons from $V_{1Gi-DREAD}$ mice ($n = 15$) and littermate controls ($n = 10$)**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>$V_{1Gi-DREAD}$</th>
<th>$V_{1Gi-DREAD}$</th>
<th>$V_{1Gi-DREAD}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>V$_{1/2}$ (mV)</strong></td>
<td>26.8 ± 1.4</td>
<td>3.8 ± 0.2</td>
<td>-9.2 ± 1.0</td>
<td>5.3 ± 0.2</td>
</tr>
<tr>
<td><strong>Slope</strong></td>
<td>24.9 ± 0.9</td>
<td>3.9 ± 0.2</td>
<td>-8.8 ± 1.2</td>
<td>5.6 ± 0.2</td>
</tr>
<tr>
<td><strong>V$_{1/2}$ (mV)</strong></td>
<td>27.1 ± 2.6</td>
<td>5.2 ± 0.3</td>
<td>-22.2 ± 0.3</td>
<td>5.2 ± 0.6</td>
</tr>
<tr>
<td><strong>Slope</strong></td>
<td>55.8 ± 1.7</td>
<td>5.9 ± 0.3</td>
<td>-19.6 ± 1.0</td>
<td>5.9 ± 0.3</td>
</tr>
</tbody>
</table>

Conduction–voltage data were fitted with a Boltzmann equation to estimate the potential at which half-maximal conductance was activated ($V_{1/2}$) and sloped. Student’s $t$ test was used to assess differences in current properties. *Indicates a significant difference between $V_{1Gi-DREAD}$ and littermate controls where $p < 0.05$, **$p < 0.01$.

Gi-DREADD expression was associated with constitutive ligand-independent suppression of Ca$^{2+}$$^+$ currents

Whereas activation of inwardly rectifying $K^+$ ($K_{IR}$) currents appears to be a common mechanism of inhibitory GPCR signaling in CNS neurons and there is evidence that $K_{IR}$ currents are present in nociceptive afferents (Gao et al., 2007; Kawano et al., 2009), there is little direct evidence of Gi–$K_{IR}$ signaling in rodent DRG neurons (McCleskey, 2000). Instead, inhibition of VGCCs (McCleskey, 2000) and AC (Taiwo and Levine, 1991) appears to be the primary mechanisms responsible for the actions of inhibitory GPCR activation. Therefore, evidence of CNO-induced activation of a $K^+$ current in our current and voltage-clamp experiments was unexpected and raised the possibility that Gi-DREADD expression may have altered normal Gi signaling in these neurons. To test this possibility, we first sought to deter-
mine whether activation of Gi-DREADD resulted in the suppression of VGCCs, as has been described for all other inhibitory GPCRs present in sensory neurons (Altier and Zamponi, 2008; Ditting et al., 2012). Tail currents were used to generate instantaneous G–V curves evoked before and after application of CNO or the μ-opioid receptor (MOR) agonist DAMGO (1 μM).

Analysis of baseline G–V data indicated that even before the application of CNO, VGCCs were smaller in neurons from V1Gi-DREADD mice compared with neurons from control littermates (Fig. 7A, B; two-way ANOVA with Holm–Sidac post hoc test). The decrease in VGCC conductance was not associated with a detectable decrease in expression of VGCC α subunits underlying N-type (CaV2.2) or P/Q-type (CaV2.1) channels (Fig. 7B, inset), which account for the majority of high-threshold current in sensory neurons (Bourinet et al., 2014). Additionally, analysis of G_{max} by sex and genotype (two-way ANOVA) revealed a significant influence of both as well as a significant interaction. Post hoc analysis shows significantly lower G_{max} in female mice compared with male mice (t = 3.06, p < 0.01) and in V1Gi-DREADD females compared with control females (t = 3.69, p < 0.01). There was no shift in the V_{1/2} of channel activation in neurons from control and V1Gi-DREADD mice (~4 ± 1.1 and ~6 ± 1.2 mV, respectively; p > 0.05, unpaired t test). There was also no difference in the voltage dependence of activation in neurons from control and V1Gi-DREADD (7.7 ± 0.2 and 8.0 ± 0.4 mV/e-fold, respectively; p > 0.05, unpaired t test). This absence of significant shifts argues against the possibility that the decrease in VGCC conductance was caused by constitutive suppression of channel activity via the G-protein βγ subunit. Nevertheless, we performed two additional analyses to further rule out this possibility. First, we analyzed the rate of current activation at 0 mV, because channels in the “sleepy” or “unwilling” state attributable to the actions of the Gβγ subunit activate more slowly (Ikeda, 1996). Consistent with the G–V data, there was no significant influence of genotype on the rate of VGCC current activation (Fig. 7C). Second, because Gβγ-mediated modulation of channel gating can be relieved with a depolarizing prepulse (to +80 mV) (Ikeda, 1996), we used a two-pulse protocol to determine whether it was possible to normalize current density in V1Gi-DREADD mice. The increase in current evoked at 0 mV after a prepulse to +80 mV was calculated as a percentage of the current evoked at 0 mV from a holding potential of −70 mV. Although there was a trend toward a larger prepulse potentiation in neurons from V1Gi-DREADD mice (22 ± 3%, n = 11 compared with 14 ± 2%, n = 16), this difference was not significant (p = 0.06; Fig. 7D).

Gi-DREADD disrupts endogenous inhibitory GPCR expression and second-messenger coupling

Despite the constitutive suppression of VGCCs in putative nociceptive DRG neurons from V1Gi-DREADD mice, the CNO-induced decrease in AP duration was also consistent with a further CNO-induced decrease in VGCC current, which could also contribute to the analgesic effects observed. Therefore, we next assessed the impact of CNO on VGCCs. For comparison, we also assessed the impact of endogenous Gi activation with the MOR agonist DAMGO. Two voltage-clamp protocols were used. The first was a voltage step to 0 mV every 5 s. Whereas the time course of current suppression could not be reasonably assessed with such a low stimulation rate, it still enabled distinguishing relatively fast signaling, which was complete within two steps or 10 s, as well as relatively slow signaling developing over tens of seconds. The second was the two-pulse protocol used to assess the extent of Gβγ-mediated suppression of VGCCs (Ikeda, 1996). The results of this analysis indicated that CNO application had no detectable influence on peak inward VGCC current, with what appeared to be a time-dependent decrease in current in neurons from both control and V1Gi-DREADD mice (Fig. 8A). Even more striking, DAMGO-induced suppression of VGCCs in neurons from V1Gi-DREADD mice in the absence of CNO was significantly attenuated compared with control mice (Fig. 8A). Furthermore, the fractional recovery of current with a prepulse in neurons from V1Gi-DREADD mice was significantly smaller than that in neurons from control littermates (Fig. 8B). The application of CNO produced no further changes in this parameter (data not shown).

Given the finding that expression of Gi-DREADD alters the cellular properties in DRG neurons, we wondered whether this change in phenotype was attributable to expression of Gi-DREADD at nonphysiological levels. To address this possibility, we measured mRNA levels of muscarinic type 4 receptors (CHRM4) by qPCR with primers that detect both mouse M4 and ectopic human M4Di receptors (i.e., the Gi-DREADD). As expected, CHRM4 levels were elevated (~13-fold) in V1Gi-DREADD mice relative to control littermates (presumably, attributable in large part to Gi-DREADD expression). Importantly, however,
levels of CHRM4 in V1Gi-DREADD mice were comparable with that of the MOR (Ct values of 24.08 ± 0.5 and 23.51 ± 0.4, respectively), suggesting that although expression of Gi-DREADD was sufficient to drive both behavioral and cellular responses, expression of the receptor would have been in a physiological range even if all of the CHMR4 mRNA recovered from V1Gi-DREADD mice was human rather than a combination of human and mouse receptors. Furthermore, expression of Gi-DREADD had no detectable influence on expression levels of other inhibitory G-protein-coupled receptors in DRG including, μ (MOR), δ (DOR), and κ (KOR) (Fig. 9A, B). It is also worth noting that relative levels of MOR, DOR, KOR, and CHRM4 in control mice were comparable with those recently described in an RNASeq-based analysis of expression levels in DRG (Goswami et al., 2014).

To assess the functional consequences of the apparent changes in endogenous Gi signaling in putative nociceptive neurons, we assessed the ability of DAMGO to prevent PGE2-induced thermal hypersensitivity. Intraplantar PGE2 (100 ng) significantly reduced PWL to 54.3 ± 3.7% (n = 8) and 73.2 ± 12.3% (n = 7) of baseline in controls and V1Gi-DREADD mice, respectively. This response to PGE2 was associated with neither a significant influence of genotype or sex (F(1,21) = 0.13, p = 0.73) nor a significant interaction between the two. The response to DAMGO was more complicated as there were significant main effects of both sex and genotype (sex: F(1,21) = 12.35, p = 0.002; genotype: F(1,21) = 6.233, p = 0.021; Fig. 9C). PGE2-induced hyperalgesia was significantly attenuated in control (n = 8) but not V1Gi-DREADD (n = 8) male mice. In contrast, DAMGO had no detectable effect in female mice regardless of genotype.
Discrimination

Based on the efficacy of DREADD technology in the CNS, we predicted that targeted expression of Gi-DREADD in nociceptive afferents would enable CNO-induced analgesia. Although there was no detectable impact of Gi-DREADD expression on baseline heat or mechanical thresholds, CNO application resulted in heat analgesia in V1Gi-DREADD mice. Furthermore, in isolated DRG neurons from V1Gi-DREADD mice, CNO decreased AP firing. The decrease in excitability was associated with a decrease in \( I_{Na} \) hyperpolarization of the resting membrane potential and activation of a low-threshold \( K^+ \) channel. CNO had no detectable influence on VGCCs in sensory neurons from V1Gi-DREADD mice. However, we observed baseline differences (CNO independent) in ion channel properties in neurons from V1Gi-DREADD mice compared with littermate controls. Specifically, there was a significant decrease in maximal TTX-R \( I_{Na} \), an increase in TTX-S \( I_{Na} \), and a decrease in VGCC conductance. There was also a loss of forskolin-induced potentiation of TTX-R \( I_{Na} \) and a decrease in the magnitude of the DAMGO-induced inhibition of VGCCs. In vivo, the loss of DAMGO effect was also detected, as DAMGO failed to block PGE2-induced hyperalgesia in V1Gi-DREADD mice.

Together, these data suggest that although it is possible to manipulate behavior via ligand-mediated Gi-DREADD activity, constitutive expression of this receptor has unintended consequences that complicate interpretation of the results.

Consistent with recent data using an AAV-based strategy to drive Gi-DREADD expression in sensory neurons (Iyer et al., 2016), initial behavioral results with the V1Gi-DREADD mice were quite promising. Given that the receptor should have no activity in the absence of CNO, we anticipated that baseline thermal and mechanical thresholds would be normal. Since pharmacological or toxin-induced ablation of TRPV1 signaling selectively renders animals unresponsive to noxious heat (Cavanaugh et al., 2009; Mishra and Hoon, 2010; Mishra et al., 2011), we predicted that silencing the TRPV1 population with CNO-induced activation of Gi-DREADDs would also result in an attenuation of the response to noxious heat, but not mechanical, stimuli. A wider pattern of expression associated with AAV-driven Gi-DREADD may explain the presence of both mechanical and thermal analgesia reported previously (Iyer et al., 2016).

Previous data indicate that peripheral administration of endogenous Gi-coupled receptor agonists does not produce
analgesia (Levine and Taiwo, 1989; Stein, 1993) but rather attenuates hyperalgesia produced by Gs-coupled receptors via inhibition of AC (Dina et al., 2005), as might occur after injury or inflammation. In contrast, spinal administration of agonists for Gi-coupled receptors is analgesic via what is thought to be inhibition of presynaptic VGCCs (Heinke et al., 2011). Thus, we anticipated that the analgesia associated with systemic administration of CNO would reflect an action at the central terminals of TRPV1cre-expressing neurons.

Unexpectedly, CNO attenuated the excitability of isolated DRG neurons. A more likely outcome was an increase in excitability caused by a decrease in large-conductance Ca2+-dependent K+ (BK) channel activity caused by CNO-induced inhibition of VGCCs (Zhang et al., 2012). Given their role in membrane potential repolarization during the falling phase of the AP (Zhang et al., 2010), a decrease in BK current would result in an increase in excitability and AP duration. The absence of a CNO-induced decrease in VGCCs may explain why there was no evidence of a secondary change in excitability or active electrophysiological properties that are dependent on BK channels. However, a reduced BK current associated with the observed CNO-independent reduction in VGCCs could account for the longer AP duration observed in neurons from V1Gi-DREADD mice.

More surprisingly was that the response to CNO included a decrease in Rm in combination with a hyperpolarization of resting membrane potential, at least in neurons from female mice. These observations suggest that the inhibitory effects of CNO were attributable, at least in part, to activation of a resting K+ conductance. Despite the detection of GIRK channels in DRG, direct evidence that these channels are engaged by endogenous Gi-coupled receptors in the absence of tissue injury is lacking. Although GIRK channel activation could account for the CNO-induced changes in the passive electrophysiological properties (decrease in Rm and hyperpolarization of resting membrane potential) observed in neurons from V1Gi-DREADD mice, their biological properties argue against this. GIRKs are hyperpolarization-activated channels that close with membrane depolarization (Lüscher and Slesinger, 2010), and they are generally not thought to contribute to AP repolarization. Furthermore, an increase in GIRK conductance associated with membrane hyperpolarization would have resulted in an increased AHP magnitude. Despite detecting GIRK channel mRNA in both control and V1Gi-DREADD ganglia, the CNO-induced decrease in excitability was associated with both a decrease in AP duration and AHP magnitude. Therefore, an alternative possibility is that Gi-DREADD is coupled to a LVA K+ channel (Gold et al., 1996b). A Gi-DREADD-mediated leftward shift in the voltage dependence of activation of such a channel would account for the CNO-induced increase in K+ conductance at resting membrane potential; the decrease in AP duration; and, as LVA K+ channels deactivate with membrane hyperpolarization, the decrease in the AHP magnitude. Accordingly, we observed an increase in LVA K+ current in neurons in which CNO was associated with a decrease in excitability.

Not only was the second-messenger signaling cascade effector system engaged by Gi-DREADD (i.e., a K+ channel) different from that engaged by endogenous Gi-coupled receptors (i.e., VGCC), but the presence of Gi-DREADD appeared to disrupt endogenous second-messenger pathways. For example, given evidence that TTX-R INa amplitude is determined by resting kinase activity (Gold et al., 1998), the smaller TTX-R INa in V1Gi-DREADD neurons is consistent with constitutive activation of Gi signaling. Although the constitutive Gi-DREADD signaling could have created a floor effect precluding the ability to detect the forskolin-induced potentiation of TTX-R INa in V1Gi-DREADD neurons, it is also possible that the absence of forskolin-induced potentiation of TTX-R INa in neurons from V1Gi-DREADD mice is attributable to the disruption of normal PKA coupling to VGSC. Consistent with this possibility, a higher concentration of forskolin (30 μM) failed to produce any detectable increase in TTX-R INa in the three neurons tested. Furthermore, the significant reduction in both the DAMGO-induced inhibition of VGCCs and the recovery of DAMGO-induced inhibition with a prepulse to +80 mV suggested that the presence of Gi-DREADD in these neurons had also disrupted the signaling of endogenous Gi-coupled GPCRs. The loss of DAMGO-induced antinociceptive effects against PGF2α-induced hyperalgesia in V1Gi-DREADD mice is also consistent with a disruption in endogenous Gi signaling. It is worth noting that the lack of DAMGO effect in female control mice may be caused by a sex difference in μ-opioid potency (Kepler et al., 1991; Craft, 2003; Mitrovic et al., 2003). Finally, the decrease in both VGCCs and VGSCs seems to compensate for the loss of normal inhibitory signaling in these neurons and could account for the relatively normal nociceptive thresholds observed in the absence of CNO.

In retrospect, the changes observed in second-messenger signaling should not have been surprising given the literature on constitutive GPCR signaling, as well as compensatory changes that emerge in response to that signaling (Liu and Prather, 2001; Fioravanti et al., 2008; Murray et al., 2010; Rank et al., 2011; Walwyn et al., 2016). Thus, there was reason to predict that changes in the balance of currents in a sensory neuron would result in changes in NaV1.7 expression. NaV1.7 seemingly plays an important role in the dynamic regulation of nociceptor excitability. Channel density is dramatically increased in the presence of inflammation (Gould et al., 1998) and at least some forms of nerve injury (Liu et al., 2012). More relevantly, there is a compensatory increase in NaV1.7 in DRG from NaV1.8 null mice (Akopian et al., 1999). Interestingly, NaV1.7 also appears to be capable of driving changes, as suggested by recent evidence that a loss-of-function mutation results in a compensatory increase in endogenous opioid expression in DRG neurons (Minett et al., 2015). The extent to which the increase in NaV1.7 contributed to the multiple changes observed in the sensory neurons from V1Gi-DREADD mice has yet to be determined. Nevertheless, given the absence of detectable changes in baseline nociceptive threshold or neuronal excitability, the changes observed seem to reflect an attempt to maintain homeostasis in the face of altered Gi signaling. In this regard, the observation that there were quantitative differences in the cellular changes observed in neurons from male and female mice underscores that it is possible to achieve the same phenotype via a variety of different mechanisms. In the context of pain and analgesia, it may not only be possible but necessary to treat men and women differently.

Before the current study, the major concern regarding clinical use of DREADDs was the small but significant proportion of CNO that is metabolized to clozapine (Jann et al., 1994; Chang et al., 1998; Löffler et al., 2012), an antipsychotic that induces gluteal release and unwanted side effects (Meltzer, 2012; Thanashahi et al., 2012; Seeman, 2014; Young et al., 2015). Consequently, new DREADD systems are under construction (Chen et al., 2015; Vardy et al., 2015). However, our results, in addition to findings that indicate differential effects between acute and chronic GiDREADD activation (Soumier and Sibille, 2014), suggest that the agonist may not be the real problem. Therefore, whereas DREADDs may ultimately prove to be an invaluable experimental tool, our results argue that significant additional refinement is required before such an approach is applicable in the clinic.
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Saloman et al. ● DREADDs as a Chemogenetic Tool to Suppress Nociception J. Neurosci., October 19, 2016 • 36(42):10769 –10781 • 10781


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