Intraarticular Adeno-Associated Virus Serotype AAV-PHP.S–Mediated Chemogenetic Targeting of Knee-Innervating Dorsal Root Ganglion Neurons Alleviates Inflammatory Pain in Mice

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Objective. Joint pain is the major clinical symptom of arthritis that affects millions of people. Controlling the excitability of knee-innervating dorsal root ganglion (DRG) neurons (knee neurons) could potentially provide pain relief. We undertook this study to evaluate whether the newly engineered adeno-associated virus (AAV) serotype, AAV-PHP.S, can deliver functional artificial receptors to control knee neuron excitability following intraarticular knee injection.

Methods. The AAV-PHP.S virus, packaged with dTomato fluorescent protein and either excitatory (Gq) or inhibitory (Gi) designer receptors exclusively activated by designer drugs (DREADDs), was injected into the knee joints of adult mice. Labeling of DRG neurons with AAV-PHP.S from the knee was evaluated using immunohistochemistry. The functionality of Gq- and Gi-DREADDs was evaluated using whole-cell patch clamp electrophysiology on acutely cultured DRG neurons. Pain behavior in mice was assessed using a digging assay, dynamic weight bearing, and rotarod performance, before and after intraperitoneal administration of the DREADD activator, Compound 21.

Results. We showed that AAV-PHP.S can deliver functional genes into ~7% of lumbar DRG neurons when injected into the knee joint in a similar manner to the well-established retrograde tracer, fast blue. Short-term activation of AAV-PHP.S–delivered Gq-DREADD increased excitability of knee neurons in vitro (P = 0.02 by unpaired t-test), without inducing overt pain in mice when activated in vivo. By contrast, in vivo Gi-DREADD activation alleviated digging deficits induced by Freund’s complete adjuvant–mediated knee inflammation (P = 0.0002 by repeated-measures analysis of variance [ANOVA] followed by Holm-Sidak multiple comparisons test). A concomitant decrease in knee neuron excitability was observed in vitro (P = 0.005 by ANOVA followed by Holm-Sidak multiple comparisons test).

Conclusion. We describe an AAV-mediated chemogenetic approach to specifically control joint pain, which may be utilized in translational arthritic pain research.

INTRODUCTION

Peripheral sensitization, manifested by an increase in the excitability of dorsal root ganglion (DRG) neurons, underlies many chronic pain pathologies such as inflammatory arthritis (1). DRG neurons display great heterogeneity, based upon both gene expression (2,3) and functional attributes (4), and this heterogeneity is further compounded by target innervation (5,6). The variation in DRG neurons offers a unique opportunity to selectively tune the excitability of a distinct subset of DRG neurons in order to provide...
pain relief with reduced side effects. For example, we have recently shown that the excitability of knee-innervating DRG neurons (identified by retrograde tracing) is increased in a mouse model of inflammatory joint pain (7) and after incubation with human osteoarthritis (OA) synovial fluid samples (8). These findings suggest that modulation of the knee-innervating DRG neuron subset (knee neurons) could help control arthritic pain.

One way of modulating neuronal excitability is to induce expression of inhibitory or excitatory designer receptors exclusively activated by exogenous chemical actuators, which by themselves do not have any endogenous effects. Modified muscarinic receptor–based designer receptors exclusively activated by designer drugs (DREADDs) is a technology based on this concept, which can increase or decrease neuronal (mostly in the central nervous system [CNS]) firing and which consequently affects a variety of behaviors (9,10), such as enhanced feeding (11) or decreased wakefulness (12). In the peripheral nervous system (PNS), activation of the inhibitory DREADD hM4D(Gi) in voltage-gated sodium channel 1.8 (NaV1.8)–expressing DRG neurons decreased knee hyperalgesia and mechanical allodynia, along with a decrease in DRG neuron firing, in mice with early experimental OA pain induced by surgical destabilization of the medial meniscus (13). This attenuation of hyperalgesia occurred at a similar level compared to the administration of 10 mg/kg morphine, suggesting that peripherally acting analgescics can be comparably potent to well-established opioids.

Similarly, activating Gαi-DREADD in transient receptor potential vanilloid 1 (TRPV1)–expressing DRG neurons (14) increased the heat pain threshold and reduced neuronal excitability in mice. Both of those studies used transgenic mice and therefore are less translatable across species due to the technical difficulties associated with targeting sensory neuron subpopulations in species without such transgenic tractability. In wild-type mice, Gαi-DREADD delivered intraneurally to the sciatic nerve via adenovirus expressed in sensory neurons (15). Importantly, however, none of these studies were specific to DRG neuron subsets innervating specific organs.

AAVs are useful tools for gene transfer that have been used for gene therapy in a variety of human diseases (16), with multiple AAV-based clinical trials currently underway for arthritis (ClinicalTrials.gov identifiers: NCT02727764 and NCT02790723) (17). AAVs can be used in conjunction with DREADD technology to selectively modulate neuronal activity of specific neuronal circuitry. Indeed, this has been achieved in the CNS (18). However, delivering genes by AAV injection into a peripheral organ to DRG neurons is challenging because of the low transduction capability of AAVs and the large anatomical distances involved in the PNS (19). A variety of AAV serotypes have shown little efficacy in transducing DRG neurons when injected subcutaneously, intramuscularly, or in an intraplantar manner in adult mice (20,21). To date, direct injection into DRG (22) or intrathecal injection (23,24) are the best ways of transducing DRG neurons. However, these routes of administration are invasive, technically complicated to perform, and do not enable transduction of neurons innervating a defined target. In the present study, we provide evidence that the PNS-specific AAV serotype, AAV-PHPS (19), can infect DRG neurons with functional cargo following injection into the knee joint. Furthermore, using the inhibitory DREADD, hM4D(Gi), as a cargo, we show that its activation normalizes the inflammatory pain–induced deficit in digging behavior in mice, which is an ethologically relevant spontaneous pain measure indicating well-being (25). Our study thus extends the use of AAV and DREADD technologies to study DRG neurons infected from a peripheral organ, which may have clinically relevant applications in the future in controlling pain pathologies.

MATERIALS AND METHODS

Animals. C57BL/6J (Envigo) mice ages 10–15 weeks (n = 30) of both sexes were used in this study. Mice were housed in groups of up to 5 in a temperature-controlled (21°C) room on a 12-hour light/dark cycle with food, water, and appropriate enrichment available ad libitum. Mice used in this study were regulated under the Animals (Scientific Procedure) Act 1986, Amendment Regulations 2012. All protocols were approved by a UK Home Office project license (P7EBFC1B1) and reviewed by the University of Cambridge Animal Welfare and Ethical Review Body.

Viruses. AAV-PHPS-CAG-ttdTomato (no. 59462-PHPS) was purchased from Addgene. AAV plasmids for DREADD viruses were also purchased from Addgene (Supplementary Table 1, on the Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41314/abstract) and packaged at the European Molecular Biology Laboratory in Rome, as previously described (26). Ten 150-mm dishes of HEK293T cells (ATCC) were triple-transfected with plasmids of AAV-PHPS, helper (Agilent), and cargo in a 1:4:1 ratio with polyethyleneimine (PEI) reagent (1:3 plasmid to PEI ratio; Sigma) (Supplementary Table 1). Three days after transfection, cells and media were collected, and this mixture was centrifuged at 3,700g at 4°C to remove debris and then concentrated by ultrafiltration using Vivatap 200 (Sartorius). The purified AAV particles were collected after running the viral concentrate through an iodixanol column (Opti-Prep density gradient medium; Alere Technologies) by ultracentrifugation (L8-70M; Beckman) at 44,400g for 2 hours at 18°C, followed by filtration using a 100-kd filter to further concentrate the samples and for buffer exchange. Viral titers (vg/ml) were measured by probing with woodchuck hepatitis virus posttranscriptional regulatory element regions (forward GGCTGTTGCGACACTGAAAT, reverse CGAAGGAGCTAGCAAGAAG) with quantitative polymerase chain reaction (qPCR) of linearized virus particles with SYBR Green as previously described (26), using a StepOnePlus.
Real-Time PCR system according to the guidelines of the manufacturer (Applied Biosystems).

**Knee injections.** All knee injections were conducted under anesthesia (100 mg/kg ketamine and 10 mg/kg xylazine, intraperitoneally) through the patellar tendon. For experiments with retrograde tracing, 1.5 μl of fast blue or titer-matched AAV-PHP.S viruses were injected intraarticularly into mouse knee joints (~4 x 10^{11} vg of AAV-PHP.S-CAG-dTomato and ~5 x 10^{11} vg of AAV-PHP.S-CAG-YFP, in which injection of 2 μl (~3 x 10^{11} vg) and 10 μl (~10^{12} vg) labeled a similar number of neurons as assessed by acute culture. For experiments involving inflammation, 7.5 μl Freund’s complete adjuvant (CFA) (10 mg/ml; Chondrex) was injected into the left knee, and Vernier calipers were used to measure knee width before and 24 hours after CFA injection (7).

**Behavioral testing.** All behavioral experiments were carried out between 10:00 AM and 1:00 PM in the presence of 2 investigators (SC and LAP). Mice were assigned randomly to control and experimental groups, and ≥2 cohorts of mice (8–12 mice per group) were assessed in each group on separate days. Mice were trained on digging and rotarod 1 day before the test day. The groups tested in this study are described below.

**Compound 21 (C21) controls.** As controls, one group of mice did not undergo knee injections. Behavioral tests (described below) were performed on these mice 20 minutes before and after intraperitoneal C21 injection (2 mg/kg diluted in sterile saline from a stock of 100 mM in ethanol; Tocris).

**Activation of Gq-DREADD.** To assess the effects of activation of Gq-DREADD, mice received an intraarticular injection of virus, and 3–4 weeks later were behaviorally tested 20 minutes before and after vehicle injections (1:100 ethanol in sterile saline) or C21 injections.

**Activation of Gi-DREADD.** To assess the effects of activation of Gi-DREADD, mice received an intraarticular injection of the virus, and 3–4 weeks later, baseline behavioral tests were conducted on mice (pre-CFA). CFA was injected into the knee joints the next day to induce inflammation. Twenty-four hours later, animals were retested 20 minutes before vehicle injections and after vehicle or C21 injections.

**Digging.** Digging behavior was measured as an assessment of spontaneous pain, as previously described (7), for 3 minutes in a standard cage with a wire lid and filled with Aspen mid 8/20 wood chip bedding (LBS Biotechnology). For training, mice were habituated in the test room in their home cages for 30 minutes, and then they were allowed to dig twice for 3 minutes with a 30-minute break in between. On each subsequent test day, mice were habituated and tested once on the 3-minute paradigm. Test sessions were video-recorded, and the digging duration was later independently coded by investigators who were blinded with regard to the conditions. The number of dig sites (burrows) was coded on test days by the investigators.

**Rotarod.** Locomotor function and coordination of mice were tested using a rotarod apparatus (Ugo Basile 7650). Mice were tested on a constant-speed rotarod at 7 revolutions per minute for 1 minute, then in an accelerating program (7–40 rpm in 5 minutes) for 6 minutes. The same protocol was used to train mice 1 day before testing. Mice were removed from the rotarod after 2 passive rotations or when they fell from the rotarod. Mice were video-recorded on test days, and 1 investigator who was blinded with regard to the conditions coded these videos for latency(s) to passive rotation or fall.

**Dynamic weight bearing.** Deficit in weight-bearing capacity is a characteristic measure of spontaneous inflammatory pain behavior, and we measured this behavior using a dynamic weight bearing device (Bioseb) in freely moving mice for 3 minutes. Animals were not trained on this device, and coding was done by 1 investigator who was blinded with regard to the conditions. In ≥1 minute and 30 seconds of the 3-minute recording, fore paw and hind paw prints were identified using the 2 highest confidence levels (based on correlation between manual software algorithm tracking) of the built-in software, ≥30 seconds of which was manually verified.

**DRG neuron culture.** Lumbar DRG (L2–L5) were collected from a subset of mice in the vehicle group 3–4 weeks after virus injections in ice-cold dissociation media (7). DRG were then enzymatically digested followed by mechanical trituration (7). Dissociated DRG neurons, thus isolated, were then plated onto poly-d-lysine– and laminin-coated glass-bottomed dishes (P35GC-1.5-14-C; MatTek) in DRG culture medium (L-15 Medium [1×] + GlutaMAX-I, 10% [volume/volume] fetal bovine serum, 24 mM NaHCO₃, 38 mM glucose, 2% penicillin/streptomycin) and maintained overnight (8–10 hours) in a humified incubator at 37°C with 5% CO₂.

**Electrophysiology.** For control experiments, DRG neurons were bathed in extracellular solution (ECS) containing 140 mM NaCl, 4 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 4 mM glucose, and 19 mM HEPES, adjusted to pH 7.4 with NaOH while recording. To investigate effects of C21 activation, neurons were incubated 10 minutes prior to the start of recording and throughout the recordings in 10 mM C21 (diluted in ECS from 100 mM stock). Only virus-transduced mice that were identified by their fluorescence upon excitation with a 572-nm LED (Cairn Research) were recorded, using patch pipettes of 5–10 MΩ (P-97 Flaming/Brown puller; Sutter Instruments) with intracellular solution containing 110 mM KCl, 10 mM NaCl, 1 mM MgCl₂, 1 mM EGTA, 10 mM
HEPES, 2 mM Na<sub>2</sub>ATP, and 0.5 mM Na<sub>2</sub>GTP, adjusted to pH 7.3 with KOH.

Action potentials (APs) were recorded in current clamp mode without current injection (to investigate spontaneous AP firing) or after stepwise injection of 80 msec current pulses from 0 to 1,050 pA in 50 pA steps using a HEKA EPC-10 amplifier (Lambrrecht) and corresponding Patchmaster software. AP properties were analyzed using Fitmaster software (HEKA) or IgorPro software (Wavemetrics) as previously described (7). Neurons were excluded from analysis if they did not fire an AP in response to current injections.

**Immunohistochemistry.** Mice with unilateral coinjections of AAV-PHP.S-tdTomato (2 μl) and fast blue (1.5 μl) were transcardially perfused with 4% (weight/volume [w/v]) paraformaldehyde (in phosphate buffered saline [PBS], pH 7.4) under terminal anesthesia (sodium pentobarbital, 200 mg/kg, intraperitoneal). L2–L5 DRG were then collected from the injected side, while L3–L4 DRG were collected from the un.injected side and postfixed in Zamboni’s fixative for 1 hour, followed by overnight incubation in 30% (w/v) sucrose (in PBS) at 4°C for cryoprotection. DRG were then embedded, snap-frozen, sectioned, and stained as previously described (7), using anti-TRPV1 antibody (1:500; AGP-118, anti–guinea pig polyclonal; Alomone) with Alexa Fluor 488–conjugated secondary antibody (1:500; 706-545-148, anti–guinea pig polyclonal; The Jackson Laboratory). Positive neurons were scored as previously described (7), using an R toolkit (https://github.com/amapruns/Immunohistochemistry_Analysis) followed by manual validation. Briefly, mean gray value (intensity) of all neurons in 1–3 sections from each DRG level of interest in each mouse was measured using ImageJ. A neuron was scored as positive for a stain if it had an intensity value >2 SDs above the average normalized minimum gray value across all sections.

**Statistical analysis.** Comparisons between 2 groups were made using the appropriate Student’s t-test (paired for repeated measures and unpaired otherwise), while 3-group comparisons were made using repeated-measures or one-way analysis of variance (ANOVA) followed by Holm-Sidak multiple comparisons tests. Chi-square tests were used to compare proportions of categorical variables. Data are presented as the mean ± SEM.

**RESULTS**

**Robust transduction of DRG neurons by knee-injected AAV-PHP.S.** AAV-PHP.S was engineered to have a higher specificity toward peripheral neurons (19), and thus we hypothesized that this AAV serotype would be able to transduce DRG neurons when injected intraarticularly into the knee joint. Consistent with our hypothesis, when AAV-PHP.S-CAG-dTomato and the commonly used retrograde tracer, fast blue, were co-injected unilaterally into 1 knee in mice (n = 3, all female), we observed both fast blue and virus labeling (Figure 1A). Comparable to findings of previous studies that used fast blue and other retrograde tracers (7,27,28), we observed a similar proportion of labeling in the lumbar DRG neurons with fast blue and AAV-PHP.S-CAG-dTomato (Figure 1B). Across L2–L5 DRG, there was ~40% co-labeling of neurons with fast blue and AAV-PHP.S-CAG-dTomato, which suggests that neither retrograde tracer is able to label the entire knee neuron population (Figure 1C and Supplementary Figure 1, Figure 1. Retrograde tracing of knee-innervating dorsal root ganglion (DRG) neurons using fast blue (FB) and an adeno-associated virus (AAV) product, AAV-PHP.S-CAG-dTomato. A, Representative images of a whole L3 DRG section showing knee neurons traced using fast blue (blue) or AAV-PHP.S-CAG-dTomato (pink), and stained using an anti–transient receptor potential vanilloid channel 1 (TRPV1) antibody (yellow). Arrowheads point to a neuron with fast blue, AAV-PHP.S, and TRPV1 colocalization; arrows point to a neuron that shows only fast blue and AAV-PHP.S colocalization. B, Percentage of neurons in L2–L5 DRG labeled with fast blue and AAV-PHP.S. C and D, Percentage of neurons showing colocalization of fast blue and AAV-PHP.S (C) and TRPV1 and AAV-PHP.S (D), expressed as a percentage of AAV-PHP.S-positive neurons. Bars show the mean ± SEM from 3 female mice.
However, area analysis of the labeled neurons suggests that similar-sized neurons are targeted by both strategies (Supplementary Figure 1). Furthermore, we observed minimum labeling in the contralateral side (Supplementary Figure 1).

Previous studies suggest that ~40% of knee neurons are TRPV1-expressing putative nociceptors (7,28). Similarly, through immunohistochemical analysis of DRG neurons, we found that ~30% of virus-labeled and fast blue–labeled neurons (mostly small-diameter neurons) express TRPV1 (Figure 1D and Supplementary Figure 1, http://onlinelibrary.wiley.com/doi/10.1002/art.41314/abstract), suggesting that viral transduction did not substantially alter expression of the nociceptive protein, TRPV1. Taken together, these findings indicate that intraarticular injection of AAV-PHP.S–CAG-dTomato in the knee joint transduces mouse DRG neurons in a similar manner to that of a routinely used retrograde tracer.

Spontaneous pain behavior is unchanged by excitatory Gq-DREADD delivered intraarticularly by AAV-PHP.S-hSyn-hM3D(Gq)-mCherry, but motor coordination deficit is provoked. Next, we tested whether AAV-PHPS can deliver functional hM3D(Gq) cargo into knee neurons via intraarticular

Figure 2. Gq–designer receptor exclusively activated by designer drugs (DREADD) activation of mouse knee neurons in vitro and in vivo. A, Representative image of neuron transduced by adeno-associated virus product AAV-PHP.S-hSyn-hM3D(Gq)-mCherry (bar = 25 μm; triangular shadow = recording electrode). B and C, Resting membrane potential and spontaneous action potential (AP) firing (B) and threshold and multiple AP firing (C) under control (ctrl) conditions (n = 19 neurons) and with Compound 21 (C21) treatment (n = 18 neurons). Data were obtained using 2 male and 3 female mice. Each symbol represents an individual neuron; bars show the mean ± SEM. D, Timeline showing when behaviors were assessed. E–J, Digging duration and number of burrows, ratio of front and rear paw weight, and rotarod behavior before and after vehicle (veh) injection (E, G, and I) or before and after C21 injection (F, H, and J). Each symbol represents an individual mouse (n = 4 male and 4 female mice); bars show the mean ± SEM. * = P < 0.05 by unpaired t-test; ** = P < 0.01 by paired t-test. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.41314/abstract.
injection, using whole-cell patch clamp on acutely dissociated neurons isolated from mice with no previous exposure to the DREADD activator C21. C21 was chosen as the DREADD activator since it has good bioavailability (57% plasma concentration 1 hour postinjection), is not converted into clozapine or clozapine N-oxide (29), and had no off-target effect in the behavioral tests conducted in naïve mice not injected with DREADDs (Supplementary Figure 2, http://onlinelibrary.wiley.com/doi/10.1002/art.41314/abstract). Gq-DREADDs couple to GqPCR pathways, and their activation causes neuronal excitation (9). Therefore, we hypothesized that when incubated with 10 nM C21, virally transduced neurons would be hyperexcitable compared to virally transduced neurons bathed in normal ECS. In accordance with this hypothesis, we observed an increased number of small- and medium-diameter (Supplementary Figure 3, http://onlinelibrary.wiley.com/doi/10.1002/art.41314/abstract), mostly nociceptive neurons (characterized by an AP half-peak duration of >1 msec and a “hump” during repolarization) firing APs without injection of current in the C21 group (vehicle group 5.3% versus C21 group 27.8%; P = 0.03 by chi-square test) (30) (Figures 2A and B and Supplementary Figure 3, http://onlinelibrary.wiley.com/doi/10.1002/art.41314/abstract).

Moreover, upon increased stepwise current injections, the AP threshold was decreased in the C21 group (P = 0.02 by unpaired t-test) (Figure 2C); no change was observed in other electrical properties measured in these neurons (Table 1). Our data also suggest that virally transduced neurons are viable, because the reported AP threshold is very similar to what we have previously observed in fast blue-positive knee-innervating neurons (31).

Based on previous studies (7,8), we hypothesized that the increased excitability of knee-innervating neurons in vitro via the Gq-DREADD system would cause pain-like behavior in mice in vivo, which was tested by assessing digging behavior (a measure of spontaneous pain as previously described [7,32]), dynamic weight bearing, and rotarod behavior (a measure of motor coordination [33]) (Figure 2D). Three weeks after virus injection into both knee joints, mice injected with vehicle or C21 (n = 4 male and 4 female mice in each group) did not show significant changes in digging behavior (mean ± SEM digging duration pre-vehicle 31.6 ± 3.7 seconds versus post-vehicle 21.3 ± 4.4 seconds; mean ± SEM digging duration pre-C21 25.2 ± 4.9 seconds versus post-C21 32.7 ± 4.7 seconds; mean ± SEM number of burrows pre-vehicle 4.6 ± 0.5 versus post-vehicle 3.5 ± 0.4; mean ± SEM number of burrows pre-C21 3.9 ± 0.7 versus post-C21 3.6 ± 0.5) (Figures 2E and F) or weight bearing (mean ± SEM front paw to rear paw weight ratio pre-vehicle 0.5 ± 0.02 versus post-vehicle 0.5 ± 0.04; mean ± SEM front paw to rear paw weight ratio pre-C21 0.4 ± 0.06 versus post-C21 0.4 ± 0.03) (Figures 2G and H).

By contrast, after injection of C21, mice showed a marked decline in their ability to remain on the rotarod (mean ± SEM pre-vehicle 273.5 ± 27.2 seconds versus post-vehicle 243.9 ± 32.7 seconds; mean ± SEM pre-C21 336.1 ± 12.9 seconds versus post-C21 249.0 ± 18.9 seconds; P = 0.002 by paired t-test), suggesting a deficit in their motor coordination (Figures 2I and J). DRG for all virus-injected mice were evaluated under a fluorescence microscope to check for viral transduction, and a subset of these DRG were further analyzed to reveal similar transduction profiles between AAV-PHPS-hSyn-Gq-DREADD-mCherry and AAV-PHPS-CAG-dTomato (i.e., different promoters do not significantly affect transduction) (Supplementary Figure 3, http://onlinelibrary.wiley.com/doi/10.1002/art.41314/abstract). Taken together, our findings show that AAV-PHPS delivers functional Gq-DREADD into knee neurons and that when virally transduced knee neurons are activated, they do not produce overt pain-like behavior in vivo but do cause a deficit in motor coordination.

Digging behavior deficits associated with inflammatory pain are reversed by inhibitory Gq-DREADD delivered intraarticularly by AAV-PHPS-hSyn-hM4D(G)-mCherry.

Intraarticular injection of CFA induced robust knee inflammation in mice (mean ± SEM control knee width pre-CFA 3.1 ± 0.03 mm versus post-CFA 3.1 ± 0.03 mm; mean ± SEM CFA knee width pre-CFA 3.1 ± 0.02 mm versus post-CFA 4.0 ± 0.05 mm; n = 24; P < 0.0001 by paired t-test) (Figure 3A) and was previously shown to increase the excitability of knee neurons innervating the inflamed knee compared to those innervating the contralateral side (7). The post-CFA knee measurements were conducted at the end of behavioral measurements, suggesting that regardless of Gq-DREADD activation, knee inflammation persisted at 24 hours post-CFA injection.

We hypothesized that incubating Gq-DREADD–expressing knee neurons from the CFA side with C21 would reverse this increased neuronal excitability. Using whole-cell patch clamp electrophysiology of small- and medium-diameter knee neurons (Supplementary Figure 4, http://onlinelibrary.wiley.com/doi/10.1002/art.41314/abstract), the percentage of CFA knee neurons firing spontaneous APs was found to be decreased after G-DREADD activation (CFA 15% versus CFA + C21 0%; P = 0.02 by chi-square test) (Figure 3C and Supplementary Figure 3, http://onlinelibrary.wiley.com/doi/10.1002/art.41314/abstract), although there

| Table 1. AP properties of mouse knee neurons intraarticularly transduced with Gq-DREADD* |
|---------------------------------|-------------------|-------------------|
|                                 | Control group     | C21 group         |
|                                 | (n = 19)          | (n = 18)          |
| RMP, mV                         | −40.8 ± 3.0       | −42.7 ± 2.8       |
| Threshold, pA                   | 415.8 ± 69.7      | 211.1 ± 51.4t     |
| HPD, msec                       | 2.6 ± 0.5         | 1.9 ± 0.3         |
| AHP peak, mV                    | 14.7 ± 2.1        | 16.8 ± 2.8        |

* Values are the mean ± SEM. AP = action potential; DREADD = designer receptor exclusively activated by designer drugs; C21 = Compound 21; RMP = resting membrane potential; HPD = half-peak duration; AHP = afterhyperpolarization. 
† P < 0.05 versus control group, by unpaired t-test.
was no change in the resting membrane potential across all conditions (Figure 3B and Table 2). Moreover, in the absence of G<sub>i</sub>-DREADD activation, CFA knee neurons had a decreased AP threshold compared to neurons from the control side, but the AP threshold of CFA and C21 treatment (n = 22 neurons) was similar to that of neurons from the control side (P = 0.005 by ANOVA followed by Holm-Sidak multiple comparisons test; Figure 3C). These results suggest that G<sub>i</sub>-DREADD activation reverses a CFA-induced increase in nociceptor excitability in vitro (Supplementary Figure 4). Other electrical properties between groups were unchanged (Table 2).

The ability of G<sub>i</sub>-DREADD to modulate pain behavior in DRG neurons is unclear, with one study showing an increase in latency to both thermal and mechanical stimuli (15) but another showing only an increase in the paw withdrawal latency to thermal stimuli (14). Nevertheless, based on the in vitro results in these studies, we hypothesized that activation of G<sub>i</sub>-DREADDs in knee neurons post-CFA injection would reverse spontaneous pain behavior.
Table 2. AP properties of mouse knee neurons intraarticularly transduced with Gq-DREADD*

|                          | Control group (n = 22) | CFA group (n = 20) | CFA + C21 group (n = 22) |
|--------------------------|------------------------|-------------------|-------------------------|
| RMP, mV                  | −42.9 ± 2.9            | −39.2 ± 2.7       | −39.5 ± 2.7             |
| Threshold, pA            | 418.2 ± 65.6†         | 242.5 ± 45.0      | 536.4 ± 66.5‡           |
| HPD, msec                | 2.4 ± 0.6              | 2.9 ± 0.5         | 2.1 ± 0.3               |
| AHP peak, mV             | 12.9 ± 1.3             | 16.5 ± 1.3        | 13.8 ± 1.6              |

*Values are the mean ± SEM. CFA = Freund's complete adjuvant (see Table 1 for other definitions).
† P < 0.05 versus CFA group, by analysis of variance (ANOVA) followed by Holm-Sidak multiple comparisons test.
‡ P < 0.01 versus CFA group, by ANOVA followed by Holm-Sidak multiple comparisons test.

in mice (Figure 3D). In the control cohort that received vehicle 24 hours after CFA injection (n = 5 male and 4 female mice), the CFA-induced decrease in digging behavior persisted compared to pre-CFA (mean ± SEM digging duration pre-CFA 29.6 ± 2.7 seconds, post-CFA 16.6 ± 4.0 seconds, post-vehicle 9.8 ± 2.8 seconds; P = 0.0005, pre-CFA versus post-vehicle) (mean ± SEM number of burrows pre-CFA 4.8 ± 0.3, post-CFA 2.9 ± 0.3, post-vehicle 2.3 ± 0.5; n = 9; P < 0.0001 by repeated-measures ANOVA [RM-ANOVA] followed by Holm-Sidak multiple comparisons test) (Figure 3E).

However, when C21 was administered to a separate cohort of mice 24 hours after CFA injection (n = 7 male and 4 female mice), there was a marked recovery in digging behavior (mean ± SEM digging duration pre-CFA 29.7 ± 4.5 seconds, post-CFA 7.8 ± 1.9 seconds, post-C21 19.0 ± 3.9 seconds; P = 0.0002) (mean ± SEM number of burrows pre-CFA, 4.5 ± 0.3, post-CFA 2.4 ± 0.3, post-C21 3.6 ± 1.4; P = 0.0005 by RM-ANOVA followed by Holm-Sidak multiple comparisons test) (Figure 3F). These findings suggest that decreasing the excitability of knee neurons via Gq-DREADD reduces inflammation-induced spontaneous pain that is associated with an increased sense of well-being demonstrated by more digging. In contrast, acute chemogenetic inhibition of knee neurons was insufficient to reverse the CFA-induced deficit in dynamic weight bearing as a percentage of body weight (mean ± SEM rear left weight bearing pre-CFA 26.2 ± 2.0%, post-CFA 10.6 ± 1.7%, post-vehicle 11.6 ± 1.5%; P < 0.0001; mean ± SEM rear left weight bearing pre-CFA 26.1 ± 1.1%, post-CFA 12.4 ± 1.4%, post-C21 13.3 ± 2.2%; P < 0.0001 by RM-ANOVA followed by Holm-Sidak multiple comparisons test) (Figures 3G and H). This may be because gait changes relating to weight bearing are more reflective of changes in joint biomechanics that are difficult to reverse with analgesics (34).

Furthermore, no change in rotarod behavior was observed following CFA-induced knee inflammation, suggesting that this model does not cause an overt change in gross motor function and similarly that Gq-DREADD activation had no effect (mean ± SEM time on rotarod pre-CFA 303.8 ± 18.3 seconds, post-CFA 315.9 ± 11.9 seconds, post-vehicle 306.6 ± 16.8 seconds; mean ± SEM time on rotarod pre-CFA 282.7 ± 17.3 seconds, post-CFA 286.4 ± 25.3 seconds, post-C21 317.4 ± 10.3 seconds) (Figures 3I and J). DRG for all virus-injected mice were evaluated under a fluorescence microscope to check for viral transduction (Supplementary Figure 4, http://onlinelibrary.wiley.com/doi/10.1002/art.41314/abstract). Taken together, our findings indicate that specific inhibition of knee neuron excitability can reverse inflammation-induced deficits in digging behavior.

**DISCUSSION**

We found that the AAV-PHPS serotype can provide retrograde cargo delivery to DRG neurons in a peripheral tissue-specific manner when injected into the mouse knee joints, without the need for invasive procedures or the requirement to generate transgenic mice. The transduction efficacy of the virus is similar to that of the widely used retrograde tracer, fast blue. Consistent with findings of other co-labeling studies (35), the ~40% colocalization of AAV-PHPS and fast blue fluorescence suggests that not all knee neurons are labeled with either tracer and that co-labeling of <100% is possibly due to their differing modes of retrograde transfer (36,37). Furthermore, we report that ~30% of labeled neurons are TRPV1+, which fits with the previously reported identity of knee-innervating neurons as a subset being ~39% TRPV1+, ~53% CGRP+, and largely isolec- tin B4–nonbinding (28,38).

Using this model, we have shown that it is possible to increase or decrease knee neuron excitability in vitro when Gq- or Gq-DREADD cargoes were delivered, respectively, by AAV-PHPS and to provide joint-specific pain control. This result can also be extended to study the role of anatomically specific neuronal excitability when exposed to a variety of stimuli or pharmacologic interventions. In vivo, we restricted our chemogenetic activation to a short duration in order to reflect acute pain, and within this timeframe we observed no spontaneous pain-like behavior with the activation of Gq-DREADD in knee neurons. We surmised that Gq-DREADD-mediated subthreshold activation (39) of the relatively low percentage of DRG neurons did not provide sufficient nociceptive input to drive change in ethologically relevant pain behavior, whereas the observed decrease in coordination suggests that we behaviorally engaged the virally transduced neurons. Furthermore, we noted that the intraarticular injection described here would transduce DREADDs to both nociceptive and non-nociceptive populations of knee neurons, and therefore, a clear nocifensive behavior might not be apparent. In other words, a limitation of this study is that
distinct subpopulations of knee neurons are not targeted, but future studies could address this once such populations have been described for the knee, as has already been conducted for the colon (5). Future studies using a repeated dosing strategy could also be employed in our system for modeling chronic pain, being cautious of the potential risk of receptor desensitization (9).

Perhaps more relevant to future clinical applications in arthritic pain is the ability of Gq-DREADDs to reverse pain behavior by decreasing neuronal excitability of knee neurons. Indeed, we have shown that Gq-DREADD activation restores a deficit in digging behavior induced by inflammatory knee pain, similar to previous studies demonstrating normalization of burrowing/digging by nonsteroidal antiinflammatory drugs (40, 41) and a peripherally restricted TRPV1 antagonist (7) after joint pain–induced depression of this behavior. This strategy can be further refined to selectively inhibit genetically specific subpopulations of knee neurons by combining Cre-inducible viruses with their corresponding Cre-expressing transgenic mouse lines, thereby providing insights into relative contributions of different knee neuron subpopulations in arthritic pain. Selectively exciting specific knee neuron subpopulations with Gq-DREADD might produce pain-like behaviors that were not observed in this study. We also observed that the CFA-induced deficit in weight bearing was not reversed following the activation of Gq-DREADD in knee neurons, consistent with a previous study demonstrating that reversal of deficits in gait changes is difficult to achieve with analgesics (34).

Although the findings of this study imply that modulating excitability of anatomically specific peripheral neurons could control arthritic pain, a number of challenges remain to be addressed before their clinical translation. Since virus transduction and expression profile is different between nonhuman primates and rodents, the expression profile of AAV-PHP.S needs to first be validated in nonhuman primates (42). Additional work is also required to engineer more PNS-specific AAVs and to optimize DREADDs (43) and their corresponding ligands (44) for increasing transduction efficiency and regulating dosing. Overall, the present study provides initial proof of concept that peripheral tissue–innervating DRG neurons can be specifically modulated by AAVs, opening the door to future studies on gene therapy in controlling arthritic pain.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Smith had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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