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## Repeated chemogenetic activation of dopaminergic neurons induces reversible changes in baseline and amphetamine-induced behaviors

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### Abstract

**Rationale**—Repeated chemogenetic stimulation is often employed to study circuit function and behavior. Chronic or repeated agonist administration can result in homeostatic changes, but this has not been extensively studied with designer receptors exclusively activated by designer drugs (DREADDs).

**Objectives**—We sought to evaluate the impact of repeated DREADD activation of dopaminergic (DA) neurons on basal behavior, amphetamine response, and spike firing. We hypothesized that repeated DREADD activation would mimic compensatory effects that we observed with genetic manipulations of DA neurons.

**Methods**—Excitatory hM3D(Gq) DREADDs were virally expressed in adult TH-Cre and WT mice. In a longitudinal design, clozapine N-oxide (CNO, 1.0 mg/kg) was administered repeatedly. We evaluated basal and CNO- or amphetamine (AMPH)-induced locomotion and stereotypy. DA neuronal activity was assessed using *in vivo* single-unit recordings.

**Results**—Acute CNO administration increased locomotion, but basal locomotion decreased after repeated CNO exposure in TH-Cre<sup>hM3Dq</sup> mice relative to littermate controls. Further, after repeated CNO administration, AMPH-induced hyperlocomotion and stereotypy were diminished in TH-Cre<sup>hM3Dq</sup> mice relative to controls. Repeated CNO administration reduced DA neuronal firing in TH-Cre<sup>hM3Dq</sup> mice relative to controls. A two-month CNO washout period rescued the decreases in basal locomotion and AMPH response.

**Conclusions**—We found that repeated DREADD activation of DA neurons evokes homeostatic changes that should be factored into the interpretation of chronic DREADD applications and their impact on circuit function and behavior. These effects are likely to also be seen in other

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neuronal systems and underscore the importance of studying neuroadaptive changes with chronic or repeated DREADD activation.

### Keywords

Chemogenetics; dopamine; basal ganglia; midbrain; plasticity; amphetamine; locomotion; stereotypic behavior; *in vivo* electrophysiology; clozapine N-oxide

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### Introduction

Chemogenetic platforms using designer receptors exclusively activated by designer drugs (DREADDs) (Armbruster et al. 2007) are widely used to evaluate causal links between neuronal activity and behavior. Compared to optogenetics and other conventional pharmacological approaches, DREADD-based platforms can activate or suppress neurons over longer time periods and are minimally invasive, yet specific and repeatable. The most popular DREADD platforms are based on engineered human muscarinic receptors that are rendered insensitive to their natural ligand acetylcholine and respond instead to the pharmacologically inert ligand clozapine N-oxide (CNO). Because of its simplicity and longer-term effects, DREADD-based chemogenetics is frequently used as a method of choice in long-term and longitudinal studies (Burnett and Krashes 2016; Roth 2016; Smith et al. 2016; Sternson and Roth 2014).

As is observed with conventional pharmacological approaches, acute versus chronic DREADD manipulations can result in different, and sometimes even opposite, effects on circuit function and behavior. While acute stimulation or inhibition has more predictable effects, chronic perturbations in neuronal activity may engage compensatory responses. For example, anxiety-like behaviors are seen only with acute but not with chronic stimulation of the dorsal raphe (Urban et al. 2016). Conversely, antidepressant-like effects are observed only with chronic but not with acute stimulation of entorhinal cortex (Yun et al. 2018). As another contrast, acute inhibition of cortical somatostatin interneurons and basal ganglia indirect pathway neurons increases measures of behavioral emotionality and motivation, respectively; whereas chronic inhibition of these neuronal populations leads to behavioral desensitization (Carvalho Poyraz et al. 2016; Soumier and Sibille 2014).

In line with the homeostatic regulations that occur with chronic perturbations of neuronal activity, we previously reported paradoxical decreases in dopaminergic (DA) transmission and amphetamine (AMPH) response in mice with genetically-ablated expression of the glutamate transporter EAAT3 (Zike et al. 2017). Reciprocally, we found that EAAT3 overexpression in DA neurons led to increased DA transmission and AMPH response (Chohan et al. 2022a). Paralleling these observations, chronic DREADD inhibition of DA neurons in postnatal mice paradoxically leads to increases in locomotor activity and stereotypy (Salesse et al. 2020). Conversely, chronic stimulation of DA neurons in an alpha-synuclein-based parkinsonian model produces a decline in motor function (Torre-Muruzabal et al. 2019). Taken together, these findings suggest the recruitment of homeostatic plasticity mechanisms during chronic manipulations of DA neurons.

Given the clinical implications of homeostatic plasticity mechanisms in chronic brain conditions, we sought to examine the impact of repeated DREADD activation of DA neurons on baseline and AMPH-induced behaviors and neuronal activity. Importantly, while psychostimulant-induced neuroadaptations are extensively characterized, to our knowledge, homeostatic adaptations following repeated DREADD activation have not been empirically tested and are assumed on *a priori* grounds. Based on our EAAT3 findings and prior DREADD studies, we hypothesized that repeated chemogenetic stimulation of DA neurons would evoke homeostatic changes resulting in diminished baseline neuronal activity and decreased AMPH sensitivity. We employed a longitudinal study design to evaluate the evolution of DREADD-related neuroadaptations on baseline and AMPH-induced behaviors. Our findings highlight the importance of considering homeostatic neuroplasticity mechanisms in the interpretation of repeated chemogenetic manipulations and may have ramifications for understanding the neurobiology of chronic brain conditions affecting the DA system.

## Methods

### Mice

All experimental studies were approved by the New York State Psychiatric Institute Institutional Animal Care and Use Committees (IACUC) in accordance with the NIH's Guide for the Care and Use of Laboratory Animals (Council 2010). TH-Cre mice (Strain No. 008601, The Jackson Laboratory) (Savitt et al. 2005) were crossed with C57BL/6J wildtype (WT) mice to obtain heterozygous TH-Cre and littermate control WT animals. After ear-tagging and genotyping, alternate animals of each genotype were assigned to experimental groups. Adult, 8-week-old, male and female mice were used for all experiments. In prior work in TH-Cre mice, we did not find sex differences in baseline or AMPH-induced locomotion and stereotypy (Chohan et al. 2020). Data were therefore pooled from both sexes for the primary analysis in the present study. Sexes of mice used in the individual experiments are listed in the 'Experimental Timeline' section. All experiments were conducted blind to genotype. Mice were group housed under a 12-hour light/dark cycle in a temperature-controlled environment, with food and water available *ad libitum*. All experiments were conducted in the light cycle.

### Experimental Timeline

**Surgeries.**—All surgeries took place on postnatal day (P) 60 (termed Day –14 of experiment). Except for the Validation cohort, mice were allowed to recover for 14 days before the commencement of behavioral and electrophysiology experiments.

**DREADD Validation.**—For Cohort 1/Validation Cohort (Fig. 1; 8 TH-Cre<sup>hM3D(Gq)</sup> (2M/6F) and 10 WT<sup>hM3D(Gq)</sup> (5M/5F)), mice were evaluated for acute 1 mg/kg CNO-induced locomotor response 4 weeks after surgeries. 1 week later, a subset of mice (3 TH-Cre<sup>hM3D(Gq)</sup> (1M/2F) and 3 WT<sup>hM3D(Gq)</sup> (2M/1F)) was evaluated for acute CNO-induced DA neuronal activation.

**Longitudinal Behavior.**—For Cohort 2/Repeated CNO Behavior Cohort (Figs. 2–3; 8 TH-Cre<sup>hM3D(Gq)</sup> (4M/4F) and 10 WT<sup>hM3D(Gq)</sup> (7M/3F)), mice were administered 1 mg/kg CNO 1x daily from P74 (termed Day 0) to P99. During this ‘CNO phase’, mice were evaluated for CNO-induced locomotion every 4 days (i.e., on Days 0, 4, 8, 12, 16), Saline-induced locomotion on Days 15 and Day 17, and AMPH-induced hyperlocomotion on Day 18. CNO was tested repeatedly to examine evolution of CNO-induced response. Saline test days were included to test for any conditioned response to injection experience, and the persistence of locomotor activity patterns from pre-injection period. Mice received CNO injections in their home cages at the conclusion of Saline and AMPH sessions.

Mice were additionally evaluated for CNO-induced stereotypic behavior 24 hrs following each of the initial three CNO locomotor test days (i.e., on Day 1, 5, 9). AMPH-induced stereotypic behavior was evaluated 1 week after the AMPH-induced locomotor assay (to allow for AMPH washout) on Days 24–25. For this assay, mice were evaluated for Saline-induced stereotypic response on Day 24 and AMPH-induced response on Day 25. As previously, mice were administered CNO injections in their home cage at the conclusion of the Saline and AMPH sessions.

To evaluate recovery of behavioral response, mice were evaluated for Saline and AMPH-induced locomotion and stereotypic behavior 4 weeks after stopping CNO treatment (i.e., on Days 53–54 and 60–61; termed as ‘Washout 1’). Finally, mice were tested for locomotor and stereotypic behaviors 4 weeks after Washout 1 (i.e., on Days 89–90 and 96–97). Also see Fig. 1e for schematic of this timeline.

**In Vivo Single-Unit Recordings.**—For Cohort 3/Repeated CNO Electrophysiology Cohort (Fig. 4; 9 TH-Cre<sup>hM3D(Gq)</sup> (4M/5F) and 7 WT<sup>hM3D(Gq)</sup> (4M/3F)), mice were administered 1 mg/kg CNO 1x daily P74–P90 (i.e., Days 0–16). DA neuron recordings were performed on P91/Day17 ~ 24 hrs after the last CNO injection. Note that this cohort was behaviorally naïve. Also see Fig. 4 for schematic of this timeline.

**Control cohorts.**—For control cohorts Cohort 4/Repeated Vehicle Behavior (Supplemental Information Figs. SI2–3; 7 TH-Cre<sup>hM3D(Gq)</sup> and 7 WT<sup>hM3D(Gq)</sup>), and Cohort 5/Repeated Vehicle Electrophysiology (Supplemental Information Fig. SI4; 6 TH-Cre<sup>hM3D(Gq)</sup> and 5 WT<sup>hM3D(Gq)</sup>), mice were tested with Vehicle using the same timelines as mentioned above. Note that these two control cohorts were independent and behaviorally naïve.

## Surgeries

Mice were anesthetized with isoflurane (induction 4–5%, maintenance, 1.5–2% wt/vol) and placed into a stereotactic apparatus (Stoelting). After making a craniotomy, a glass capillary attached to a Nanoject (Drummond) was slowly lowered into the midbrain (AP –3.15, ML +/-0.8, DV –4.0 mm) (Chohan et al. 2022a) and 0.3 ul of undiluted AAV-hSyn-DIO-hM3D(Gq)-mCherry (Addgene, Cat No. 44361-AAV5, titer  $7 \times 10^{12}$  vg/mL) (Krashes et al. 2011) was delivered at a flow rate of 0.1 ul/min. The glass capillary was left in place for 10 min to facilitate diffusion into the brain tissue and to minimize backflow. After 10 min, the

capillary was slowly retracted, and the skin was closed with sutures (Henry Schein). Mice were provided two weeks for full recovery and virus expression.

## Behavior

**Open-field locomotion.**—Two weeks after virus expression, mice were placed in an open-field chamber (SmartFrame Open Field System, Kinder Scientific) measuring 40.6 cm long and 40.6 cm wide fitted with 32 infrared photo-beams (16X & 16Y) and connected to the Motor Monitor Software (Kinder Scientific). After an exploratory period of 30 min, mice were removed from the chambers and administered an intraperitoneal (IP) dose of either 1 mg/kg CNO, 0.9% saline, or 3 mg/kg AMPH, depending on the test session (Fig. 1e). Mice were then returned to the chambers, and locomotion was monitored over the next 60 min. Locomotor activity was recorded under bright ambient light conditions.

**Stereotypic behavior.**—Twenty-four hours after each of the initial three CNO open-field locomotion test days, mice were evaluated for CNO-induced stereotypic behavior. Stereotypic behavior was assessed in shoebox cages (30.5 cm L x 19.7 cm W x 16.5 cm H) that were distinct from the wedge-shaped, Optimouse home cages (34.3 cm L x 29.2 cm W (front) x 15.5 cm H), and the open field chambers (40.6 cm L x 40.6 cm W). Mice were also evaluated for AMPH-induced stereotypic behavior one week after each of the three AMPH open-field tests; for this, mice were administered 0.9% saline (Saline) the first day and 8 mg/kg AMPH the following day. Briefly, following 30 min of acclimation, mice were administered an IP dose of 1 mg/kg CNO, Saline, or 8 mg/kg AMPH, depending on the test session (Figs. 1e, 3a). Mice were then left unperturbed in the shoebox cages for another 90 mins. Mouse behavior was recorded using a video camera (Sony Handycam Flash Memory Camcorder, HDRCX405/B) for 2 min each at 50- and 80-min (termed T50 and T80) post-drug administration. Mice were administered their daily dose of CNO in the home cages at the conclusion of the Saline- and AMPH-induced stereotypic behavior test sessions. Two trained video observers, blind to genotype and treatment, manually recorded the time spent in stationary shuffling, licking and sniffing-like stereotypy, as previously described (Chohan et al. 2020; Chohan et al. 2022a; Zike et al. 2017).

## *In Vivo* Single-Unit Recordings

Anesthetized mice (chloral hydrate, induction 600 mg/kg, maintenance 250 mg/kg, IP) were placed into a stereotactic apparatus (Kopf) and a glass capillary (World Precision Instruments; tip diameter 1  $\mu$ m; impedance 8-10 M $\Omega$ ) filled with 2M NaCl was slowly lowered into the ventral tegmental area (VTA) (AP  $-3.15$ , ML  $\pm 0.8$ , DV  $-4.0$ mm) to detect spontaneously active DA neurons (Chohan et al. 2022a; Gilani et al. 2014). DA neurons were identified by the broad action potential width ( $>3$  msec) and tri-phasic waveform (Grace and Bunney 1984; Ungless et al. 2004). Bursts were defined as per the classical criteria of Grace and Bunney (Grace and Bunney 1984) where an onset of burst is marked by an ISI  $< 80$  msec and its offset is marked by the first spike that is preceded by an ISI  $> 160$  msec. From this starting point, the VTA was sampled in six locations spaced 0.15 mm apart and arranged in a  $2 \times 3$  spaced grid moving in a clockwise direction. Starting locations were counterbalanced across mice and groups. Neuronal activity was amplified and filtered (1000 x gain, 100-10K Hz band pass) and was fed to computer interface with an

acquisition and analysis software (NeuroScope V2) for offline analysis. Inter-spike interval (ISI) distributions, burst properties and firing rates for each DA neuron were quantified.

## Histology

Mice were perfused with phosphate-buffered saline (PBS), followed by 4% (wt/vol) paraformaldehyde (PFA) (in PBS). The brains were extracted and post-fixed in PFA for 6 hours and then cryoprotected by transferring to ascending concentrations (10, 20, 30%) of sucrose (in PBS) for 24 hours each at 4°C. 40 µm thick coronal sections spanning the midbrain region were cut on a cryostat. Sections were collected in PBS. After three 5 min washes in PBS, sections were blocked with 10% normal goat serum and 0.3% Triton X-100 in PBS for 2 hours at room temperature (RT). Sections were then incubated in 10% normal goat serum and 0.3% Triton-X with primary anti-TH (dilution 1:1000, mouse monoclonal, Sigma, T2928) and anti-RFP antibody (dilution 1:500, rabbit polyclonal, Rockland, 600-401-379) in PBS for 20 hours at 4°C. After three 5 min washes in PBS, sections were incubated with Alexa-conjugated secondary antibodies (dilution 1:500, goat anti-mouse, Alexa-Fluor 488, Life Technologies, A-11029; goat anti-rabbit, Alexa-Fluor 594, Abcam, ab150080) for 1 hour at RT. After three more steps of washing in PBS, sections were mounted on a glass slide and cover-slipped. Sections were visualized using a computer assisted morphometry system consisting of a Zeiss Axioplan2 microscope with an internal Z drive, fitted with a Ludl XY motorized stage and 1300 × 1100 pixel digital video camera (manufactured exclusively for Microbrightfield, Inc., VT) and images were captured using NeuroLucida software (MBF Biosciences).

## Drugs

CNO (Tocris Bioscience) was dissolved in 0.25% DMSO / 0.9% sterile saline (termed 'Vehicle'). D-amphetamine (AMPH) (Sigma-Aldrich) was dissolved in 0.9% sterile saline (termed 'Saline'). Mice were administered Saline during the Saline test sessions in the CNO phase. CNO was administered in the home cage at the conclusion of the Saline/AMPH sessions. Control groups (Supplemental Information Fig. SI2–3) that were repeatedly treated with Vehicle similarly received Saline during the Saline sessions followed by Vehicle in the home cage at the conclusion of the session. All drugs were administered IP at a volume of 10 ml/kg.

## Statistical analysis

Data was analyzed with GraphPad Prism (version 9.0.0, GraphPad Software, San Diego, CA, USA). Locomotor activity, expressed as horizontal distance traveled (cm), was collected in 5-min bins and analyzed using 2-way repeated-measures (RM) ANOVA. Area Under the Curve (AUC) was calculated and compared using three-way or two-way RM ANOVA. Stereotypy was analyzed using three-way or two-way RM ANOVA. For *in vivo* physiology, ISI distributions were compared using lognormal curve fit. All other physiology data was compared using unpaired two-tailed *t* tests. Multiple comparisons were evaluated by Sidak's or Tukey's post hoc tests. All data are reported as the mean ± SEM (standard error of mean). Schematics were created with [BioRender.com](https://BioRender.com).

## Results

### Validation of DREADD-based activation of dopaminergic neurons

We first validated DREADD-based activation of DA neurons. AAVs carrying the excitatory DREADD hM3D(Gq) were bilaterally injected into the midbrain of TH-Cre (TH-Cre<sup>hM3D(Gq)</sup>) and WT (WT<sup>hM3D(Gq)</sup>) mice on postnatal day (P) 60 (Fig. 1a). Four weeks following surgery, mice were acutely administered 1 mg/kg of CNO as locomotor activity was examined. As expected, increased locomotor activity was observed over the hour following CNO (two-way RM ANOVA, time x genotype interaction  $F(11, 176) = 26.89$ ,  $P < 0.0001$ , genotype  $F(1, 16) = 68.83$ ,  $P < 0.0001$ , Fig. 1b). We then used *in vivo* electrophysiology to functionally validate hM3D(Gq) receptor expression specifically in DA neurons in a sub-set of these animals. In anesthetized mice, acute IP CNO (1 mg/kg) administration increased spike firing frequency (two-way ANOVA, time x genotype interaction  $F(1919, 7583) = 1.918$ ,  $P < 0.0001$ , genotype  $F(1, 7583) = 6585$ ,  $P < 0.0001$ , Fig. 1c–d) for DA neurons in TH-Cre<sup>hM3D(Gq)</sup> mice in comparison to WT<sup>hM3D(Gq)</sup> controls. Having functionally validated hM3D(Gq) receptor activation in DA neurons, we next subjected naïve cohorts of TH-Cre<sup>hM3D(Gq)</sup> and WT<sup>hM3D(Gq)</sup> to a series of behavioral pharmacology experiments to test the impact of repeated DREADD activation (Fig. 1e).

### Repeated CNO administration results in diminished baseline and AMPH-induced locomotion in TH-Cre<sup>hM3D(Gq)</sup> mice that is reversed after CNO washout

A two-way RM ANOVA was performed on locomotor data grouped into 5 min bins. We observed robust increases in locomotor activity in response to CNO administration in TH-Cre<sup>hM3D(Gq)</sup> mice on Day 0 (two-way RM ANOVA, time x genotype interaction  $F(11, 176) = 33.64$ ,  $P < 0.0001$ , genotype  $F(1, 16) = 202.6$ ,  $P < 0.0001$ , Fig. 2a). The ability of CNO to increase locomotor activity in TH-Cre<sup>hM3D(Gq)</sup> mice remained strong across CNO test days (Day 4, interaction  $F = 31.56$ ,  $P < 0.0001$ , genotype  $F = 373.8$ ,  $P < 0.0001$ ; Day 8, interaction  $F = 22.90$ ,  $P < 0.0001$ , genotype  $F = 209.9$ ,  $P < 0.0001$ ; Day 12, interaction  $F = 40.17$ ,  $P < 0.0001$ , genotype  $F = 61.81$ ,  $P < 0.0001$ ; Day 16, interaction  $F = 34.72$ ,  $P < 0.0001$ , genotype  $F = 58.99$ ,  $P < 0.0001$ , Figs. 2b–d, f).

Expression of hM3D(Gq) did not impact baseline locomotion, measured over the 30 minute period immediately before IP injection of CNO, in TH-Cre mice on Day 0 (time x genotype interaction  $F(5, 80) = 1.057$ ,  $P = 0.3907$ , genotype  $F(1, 16) = 0.2731$ ,  $P = 0.6084$ , Fig. 2a), supporting previous work showing unchanged baseline locomotion in TH-Cre mice (Chohan et al. 2020). Strikingly, repeated administration of CNO for ~ 2 weeks significantly reduced baseline locomotion in TH-Cre<sup>hM3D(Gq)</sup> mice compared to WT<sup>hM3D(Gq)</sup> control animals (Day 4, interaction  $F = 0.7618$ ,  $P = 0.5800$ , genotype  $F = 1.969$ ,  $P = 0.1797$ ; Day 8, interaction  $F = 0.5849$ ,  $P = 0.7115$ , genotype  $F = 0.1887$ ,  $P = 0.6698$ ; Day 12, interaction  $F = 0.3309$ ,  $P = 0.8929$ , genotype  $F = 12.52$ ,  $P = 0.0027$ , Figs. 2b–d). Starting on Day 12, a decrease in baseline locomotion in TH-Cre<sup>hM3D(Gq)</sup> mice was observed on every test day for the remainder of the CNO phase (Day 15, interaction  $F = 0.6144$ ,  $P = 0.6891$ , genotype  $F = 24.95$ ,  $P = 0.0001$ ; Day 16, interaction  $F = 0.08453$ ,  $P = 0.9945$ , genotype  $F = 43.93$ ,  $P < 0.0001$ ; Day 17, interaction  $F = 1.024$ ,  $P = 0.4093$ , genotype  $F = 25.49$ ,  $P = 0.0001$ ; Day 18, interaction  $F = 2.056$ ,  $P = 0.0797$ , genotype  $F = 32.86$ ,  $P < 0.0001$ , Figs. 2e–h).

Repeated CNO treatment also significantly diminished locomotor hyperactivity in response to AMPH (3 mg/kg) in TH-Cre<sup>hM3D(Gq)</sup> mice compared to WT<sup>hM3D(Gq)</sup> control mice (Day 18, interaction  $F(11, 176) = 1.491$ ,  $P = 0.1382$ , genotype  $F(1, 16) = 6.459$ ,  $P = 0.0218$ , Fig. 2h).

The decrease in baseline locomotion in TH-Cre<sup>hM3D(Gq)</sup> mice was still present following a CNO-free, one month washout period (Day 53, interaction  $F = 0.7212$ ,  $P = 0.6094$ , genotype  $F = 8.849$ ,  $P = 0.0089$ ; Day 54, interaction  $F = 0.8374$ ,  $P = 0.5271$ , genotype  $F = 5.220$ ,  $P = 0.0363$ , Figs. 2i–j); however, this effect was not maintained after two months (Day 89, interaction  $F = 0.8703$ ,  $P = 0.5052$ , genotype  $F = 2.352$ ,  $P = 0.1460$ ; Day 90, interaction  $F = 0.9452$ ,  $P = 0.4570$ , genotype  $F = 2.098$ ,  $P = 0.1680$ , Figs. 2k–l). No significant genotype difference in AMPH-induced hyperlocomotion was seen following one month or two months of washout (Day 54, interaction  $F = 1.123$ ,  $P = 0.3458$ , genotype  $F = 1.723$ ,  $P = 0.2078$ ; Day 90, interaction  $F = 0.3552$ ,  $P = 0.9709$ , genotype  $F = 0.2449$ ,  $P = 0.6279$ , Figs. 2j,l). Area under the curve (AUC) analysis of the pre- and post-drug periods confirmed the time series analyses (Supplemental Information Fig. SI1). Importantly, repeated administration of vehicle did not impact either baseline locomotion or locomotor hyperactivity induced by AMPH in TH-Cre<sup>hM3D(Gq)</sup> animals (Supplemental Information Fig. SI2), indicating that decreases in baseline and AMPH response were not due to transgene effects.

### Repeated CNO administration induces decreases in AMPH-induced stereotypy in TH-Cre<sup>hM3D(Gq)</sup> mice that is rescued after CNO washout

We also tracked shuffling, licking, and sniffing-like stereotypies, as previously described (Chohan et al. 2020; Chohan et al. 2022a; Zike et al. 2017), induced by CNO and AMPH by performing stereotypy tests 24 hours following each of the initial three CNO open-field tests and 1 week following each of the three AMPH open-field tests (Figs. 1e, 3a). Repeated administration of CNO over a 10-day period did not produce stereotypic behavior in TH-Cre<sup>hM3D(Gq)</sup> and WT<sup>hM3D(Gq)</sup> mice (Fig. 3b), likely because locomotion was the principal behavior induced at 1 mg/kg dose of CNO (Fig. 2). Three-way ANOVA of stereotypic behavior after repeated CNO showed that there were no main effects of day ( $F(2, 32) = 2.122$ ,  $P = 0.1364$ ), genotype ( $F(1, 16) = 2.156$ ,  $P = 0.1614$ ), or time ( $F(1, 16) = 0.9629$ ,  $P = 0.3411$ ), and no three-way interaction ( $F(2, 32) = 0.0008$ ,  $P = 0.9992$ ), day-by-genotype interaction ( $F(2, 32) = 2.156$ ,  $P = 0.1323$ ), or time-by-genotype interaction ( $F(1, 16) = 0.1250$ ,  $P = 0.7283$ ).

Previous research has shown that stereotypic behavior dominates over locomotion after mice are challenged with a high dose of AMPH (Baldan et al. 2014; Yates et al. 2007). Repeated CNO administration decreased stereotypic behavior produced by administration of 8 mg/kg AMPH in TH-Cre<sup>hM3D(Gq)</sup> mice compared to WT<sup>hM3D(Gq)</sup> control animals (Fig. 3c). There were significant effects of treatment ( $F(1, 16) = 214.0$ ,  $P < 0.0001$ ), and genotype ( $F(1, 16) = 14.57$ ,  $P = 0.0015$ ), and an interaction between treatment and genotype ( $F(1, 16) = 16.85$ ,  $P = 0.0008$ ), based on three-way ANOVA. Sidak's post hoc comparisons showed that AMPH-treated TH-Cre<sup>hM3D(Gq)</sup> mice displayed lower stereotypy than WT<sup>hM3D(Gq)</sup> controls at both the T50 ( $P = 0.0001$ ) and T80 ( $P < 0.0001$ ) time points; whereas there were no



differences between the saline-treated TH-Cre<sup>hM3D(Gq)</sup> and WT<sup>hM3D(Gq)</sup> groups ( $P > 0.99$ ). Importantly, repeated administration of vehicle did not impact AMPH-induced stereotypic behavior in TH-Cre<sup>hM3D(Gq)</sup> animals (Supplemental Information Fig. S13), indicating that the differences in stereotypic behavior after AMPH administration were mediated by repeated DREADD activation.

The observed decreases in stereotypic behavior in TH-Cre<sup>hM3D(Gq)</sup> mice after repeated CNO administration was not sustained at one month of washout (interaction  $F = 0.09881$ ,  $P = 0.7576$ , genotype  $F = 0.05807$ ,  $P = 0.8128$ , Fig. 3d). Similar results were observed following two months of washout (interaction  $F = 1.205$ ,  $P = 0.2909$ , genotype  $F = 0.2701$ ,  $P = 0.6114$ , Fig. 3e). Finally, three-way ANOVA analysis to determine the effects of phase, treatment and genotype revealed a significant 3-way interaction ( $F(2, 26) = 4.311$ ,  $P = 0.0242$ ), phase-by-genotype interaction ( $F(2, 32) = 4.973$ ,  $P = 0.0132$ ), phase-by-treatment interaction ( $F(2, 26) = 5.673$ ,  $P = 0.0090$ ), and main effects of phase ( $F(2, 32) = 8.896$ ,  $P = 0.0008$ ), treatment ( $F(1, 16) = 434.7$ ,  $P < 0.0001$ ), and genotype ( $F(1, 16) = 4.745$ ,  $P = 0.0447$ ). Tukey's post hoc comparisons showed that TH-Cre<sup>hM3D(Gq)</sup> mice displayed lower levels of stereotypy in the CNO phase ( $P = 0.0001$ ) but showed comparable stereotypy to WT<sup>hM3D(Gq)</sup> mice after one month ( $P > 0.99$ ) and two months ( $P = 0.99$ ) of washout, indicating that decreases in AMPH-induced stereotypic behavior after repeated CNO were rescued after washout.

### Repeated CNO administration is associated with decreased spike firing of dopaminergic neurons in TH-Cre<sup>hM3D(Gq)</sup> mice

We next sought to evaluate whether the altered behavior following repeated CNO administration might be associated with altered spike firing of DA neurons. We used *in vivo* single-unit recordings to record spontaneous spike firing patterns from the midbrain of anesthetized TH-Cre<sup>hM3D(Gq)</sup> and WT<sup>hM3D(Gq)</sup> mice following 16 days of repeated CNO administration (Fig. 4a), the same regimen that produced diminished behavior in TH-Cre<sup>hM3D(Gq)</sup> mice. DA neurons were identified using the established criteria of long duration ( $>3$  msec), triphasic action potential waveform and tonic irregular firing with intermittent burst patterns (Chohan et al. 2022a; Gilani et al. 2014; Ungless et al. 2004). Bursts were defined using the classic method in which burst onset is marked by two spikes with an ISI  $\approx 80$  ms and offset is marked by a subsequent ISI  $\approx 160$  ms (Grace and Bunney 1984; Ungless and Grace 2012).

Strikingly, repeated CNO treatment produced a right-shift in inter-spike interval (ISI) distribution in TH-Cre<sup>hM3D(Gq)</sup> mice relative to WT<sup>hM3D(Gq)</sup> controls ( $F(1, 1610) = 284.6$ ,  $P < 0.0001$ , Fig. 4b). Using a log-normal curve fit of the ISI distribution, the geometric mean ISI was significantly increased in TH-Cre<sup>hM3D(Gq)</sup> mice compared to WT<sup>hM3D(Gq)</sup> mice (unpaired  $t$  test,  $t = 2.244$ ,  $P = 0.0416$ ). Repeated CNO treatment had a significant effect on the pattern of activity, reducing the number spikes fired per burst in TH-Cre<sup>hM3D(Gq)</sup> mice ( $t = 2.498$ ,  $P = 0.0256$ , Fig. 4c). Further evaluation of ISIs revealed significantly decreased average burst ISI ( $t = 2.516$ ,  $P = 0.0247$ , Fig. 4d) but unaffected average before-burst ISI ( $t = 0.9299$ ,  $P = 0.3682$ , Fig. 4e) in TH-Cre<sup>hM3D(Gq)</sup> mice. No between-genotype differences were observed in burst frequency ( $t = 1.395$ ,  $P = 0.1849$ , Fig. 4f) or percentage

of total spikes fired in bursts ( $t = 1.110$ ,  $P = 0.2855$ , Fig. 4g). Overall, repeated CNO administration decreased firing rate in TH-Cre<sup>hM3D(Gq)</sup> animals ( $t = 2.339$ ,  $P = 0.0347$ , Fig. 4h). Importantly, repeated administration of vehicle for 16 days did not alter ISI distribution or spike firing patterns in TH-Cre<sup>hM3D(Gq)</sup> mice relative to WT<sup>hM3D(Gq)</sup> controls (Supplementary Information Fig. SI4).

## Discussion

Using a longitudinal study design, we demonstrate that repeated pharmacogenetic stimulation of DA neurons in the midbrain leads to decreases in basal locomotion as well as AMPH-induced stereotypic behavior. These changes are accompanied by decreases in basal spike firing activity of DA neurons. Specifically, repeated daily DREADD activation induces a right-shift of the inter-spike interval distribution and leads to a reduced number of spikes fired in bursts. Finally, we show that the observed behavioral changes can be rescued after a CNO washout period, indicating that the induced changes are reversible.

There are also some important caveats for our results. First, we were interested in understanding the longitudinal impact of CNO administration and washout on AMPH response, but we cannot exclude the possibility that repeated AMPH administration resulted in sensitization that could contribute to the observed rescue of locomotor and stereotypy response in the washout phase. Future studies could use separate cohorts of mice to evaluate the impact of CNO washout on locomotor and stereotypy response in naïve mice (i.e., not previously exposed to AMPH). Further, because same open field chambers were used for each locomotor session, we cannot fully rule out the role of contextual learning processes in our observed phenotypes. However, while we might expect some level of associative learning to have occurred, the literature on repeated exposure to DA agonists would predict increased, not decreased, activity during the pre-injection period and a conditioned response to saline injections, based on the well-known role of DA in stimulus-reward learning and previous research showing increases in anticipatory (Ma et al. 2010; Mohawk et al. 2013; Sequeira-Cordero and Brenes 2021; Shibata et al. 1994; Shibata et al. 1995; Weiss et al. 1992; Zweifel et al. 2008), and conditioned activity (Alam 1981; Anagnostaras and Robinson 1996; Bevins and Peterson 2004; Borgkvist et al. 2008; Brabant et al. 2003; Browman et al. 1998; Damianopoulos and Carey 1992; Drew and Glick 1988; Gold and Koob 1989; Gold et al. 1988; Hall et al. 2008; Itzhak 1997; Mazurski and Beninger 1987; Michel et al. 2003; Rauhut and Bialecki 2011; Schiff 1982; Tilson and Rech 1973; Tirelli and Terry 1998; Vezina and Leyton 2009), and DA neuron firing (Henry et al. 1989; Lodge and Grace 2008; White and Wang 1984). Importantly, our finding of reduced DA neuron firing in mice that were previously administered CNO in their home cages, suggests that homeostatic adaptations in DA neurons contribute, at least in part, to our observed phenotypes. We also did not evaluate DA neuron firing after CNO washout; although the rescue of basal locomotion would suggest that DA transmission was likely rescued. Finally, we used injection of hM3D(Gq) virus in wildtype animals as a control for the effects of viral injection without Cre-mediated recombination and DREADD expression; however, we did not use a control virus in TH-Cre mice to control for the effects of genotype. This concern is ameliorated somewhat because we focused on change from baseline within animals using a longitudinal design, rather than focusing on cross-sectional comparisons within genotype.

Additionally, our previous work showed equivalent baseline and AMPH-induced locomotion in TH-Cre mice compared to wildtype C57BL/6 controls (Chohan et al. 2020).

It is noteworthy that repeated DREADD activation of DA neurons produced changes in behavior and neuronal activity that are in many respects opposite to the changes that are produced with repeated exposure to drugs of abuse. For example, we did not observe sensitization (Robinson and Berridge 1993; Steketee and Kalivas 2011; Stewart and Badiani 1993; Vanderschuren and Kalivas 2000; Vezina 2004; Vezina and Leyton 2009), a well-known property of amphetamine (Pierce and Kalivas 1997; Robinson and Becker 1986; Robinson et al. 1982; Segal and Mandell 1974; Vanderschuren et al. 1999) and cocaine (Guan et al. 1985; Jackson and Nutt 1993; Kalivas and Duffy 1990; Shuster et al. 1977), and which is thought to be mediated, at least in part, by drug-induced adaptations in DA neurons (Carlezon and Nestler 2002; Robinson and Berridge 1993; Wolf 1998). Furthermore, baseline locomotion decreased after repeated DREADD activation, which contrasts with the increased locomotion that is found with repeated exposure to drugs of abuse (Ma et al. 2010; Mohawk et al. 2013; Sequeira-Cordero and Brenes 2021; Shibata et al. 1994; Shibata et al. 1995; Weiss et al. 1992; Zweifel et al. 2008). There was also no conditioned response induced by saline after repeated CNO, which contrasts with the conditioned hyperactivity that is induced with repeated administration of DA enhancer drugs (Alam 1981; Anagnostaras and Robinson 1996; Bevins and Peterson 2004; Borgkvist et al. 2008; Everitt and Wolf 2002; Gold et al. 1988; Hall et al. 2008; Itzhak 1997; Mazurski and Beninger 1987; Michel et al. 2003; Rauhut and Bialecki 2011; Schiff 1982; Tilson and Rech 1973). Repeated CNO did not augment the effects of acute amphetamine, and instead diminished it, ruling out cross-sensitization, which we had also expected to see based upon previous DA agonist experiments (Bonate et al. 1997; Ferrario and Robinson 2007; Holly et al. 2012; Itzhak and Martin 1999; Liu et al. 2007; Schenk et al. 1991; Yang et al. 2003). Importantly, repeated exposure to the DREADD agonist led to decreased DA neuron firing, which contrasts with the increased neuronal activity that is observed with repeated administration of drugs of abuse (Ackerman and White 1990; Borgland et al. 2004; Clark and Overton 1998; Henry et al. 1989; Kalivas and Duffy 1993; Lodge and Grace 2008; Ungless et al. 2001; White and Wang 1984; Zhang et al. 1997). Notably, neuroadaptations underlying DA agonist-induced sensitization response are driven by separate and to some extent independent pre- and postsynaptic mechanisms. Recent work has implicated non-DA neurons in the midbrain and postsynaptic adaptations in striatal medium spiny neurons in the mediation of sensitization response that is associated with repeated exposure to amphetamine and cocaine (Beutler et al. 2011; Fourgeaud et al. 2004; Heusner and Palmiter 2005; Lee et al. 2006; Robinson and Kolb 1997). It thus is plausible that the selective activation of DA neurons with DREADDs leads to neuroadaptations that differ from the homeostatic adaptations that are produced by drugs of abuse.

With regard to our primary findings, the decreased basal locomotion in TH-Cre<sup>hM3D(Gq)</sup> mice is surprising because the acute response to CNO did not change over the course of the experiment, which suggests that motor capacity is intact in these animals. Our data are, however, consistent with recent studies that have reported paradoxical increases in basal locomotion following repeated DREADD inhibition (using twice-daily CNO injections) of DA neurons from P14 to P30 (Salesse et al. 2020), as well as aggravation of

diminished motor function following repeated DREADD excitation of the substantia nigra (using once-daily CNO injections over 4 weeks) in an alpha-synuclein-based parkinsonian model (Torre-Muruzabal et al. 2019). These independent reports suggest recruitment of homeostatic mechanisms to offset chronic DREADD-induced changes in DA transmission. Our *in vivo* single-unit recording data showing decreased DA neuron firing suggest that presynaptic changes mediate at least some of the change in baseline locomotion, but other pre- or post-synaptic mechanisms could also be involved. Future studies examining the impact on presynaptic transmission, DA content, release, and post-synaptic DA receptor function will be important for revealing the molecular mechanisms underlying decreased basal locomotion and spike firing.

While a significant main effect of genotype on AMPH-induced hyperlocomotion was found via two-way RM ANOVA of time-series data in mice that were repeatedly treated with CNO, a lack of treatment-by-genotype interaction via AUC analysis indicates that this was likely driven by decreased basal locomotion. On the contrary, the paradoxical decreases in AMPH-induced stereotypic behavior in TH-Cre<sup>hM3D(Gq)</sup> mice were robust, as evidenced by a significant treatment-by-genotype interaction via three-way ANOVA and full rescue of the diminished behavior after CNO washout, indicating reduced AMPH sensitivity in TH-Cre<sup>hM3D(Gq)</sup> mice. The paradoxical decreases in AMPH-induced behavior in TH-Cre<sup>hM3D(Gq)</sup> mice that were repeatedly treated with CNO parallels what we observed in EAAT3 knockdown and overexpressing mice previously, with a loss or gain of EAAT3 paradoxically leading to decreased or increased AMPH response (Chohan et al. 2022a; Chohan et al. 2022b; Zike et al. 2017), respectively, likely due to homeostatic changes in glutamate transmission after chronic alterations in glutamate exposure. Intriguingly, homeostatic regulations have been posited to underlie the decreased excitatory postsynaptic transcripts in obsessive-compulsive disorder (OCD) (Piantadosi et al. 2019), potentially convergent with recent data pointing to DA's role in gating of OCD-like grooming behavior (Xue et al. 2022). While we did not evaluate grooming behavior in our mice, our AMPH data are consistent with the suppression of grooming behavior that follows acute optogenetic inhibition of DA neurons (Xue et al. 2022).

Most stimulant drugs increase DA firing and release when administered acutely but lead to blunted DA transmission after repeated exposure (Bloomfield et al. 2016; Martinez et al. 2005; Martinez et al. 2012; Morikawa and Morrisett 2010; Nestler 2005; Subramanian and Dani 2015; Wang et al. 2012). Some studies have posited decreased vesicular monoamine transporter binding and structural plasticity as possible mechanisms (Gilman et al. 1998; Little et al. 2003; Narendran et al. 2012), with the latter also induced by chronic chemogenetic manipulations of DA neurons (Bian et al. 2022). Based on our chemogenetic findings, decreased DA neuron spike firing could be an additional mechanism that contributes to the blunted DA transmission that is observed following repeated stimulant use (Ashok et al. 2017; Martinez et al. 2007; Volkow et al. 2014; Volkow et al. 1997; Volkow et al. 2007).

It is intriguing that chemogenetic modulation of DA neurons would impact response to AMPH, which is conventionally believed to increase DA release independent of neuronal firing via reverse transport mechanisms (Freyberg et al. 2016; Jones et al. 1998; Kahlig et

al. 2005; Sulzer et al. 1995). Recent *in vivo* studies point to an additional action potential-dependent mechanism of AMPH effects on behavior (Avelar et al. 2013; Covey et al. 2016; Daberkow et al. 2013; Ramsson et al. 2011). For instance, AMPH increases frequency, amplitude, and duration of phasic DA release (Daberkow et al. 2013) and can improve learning and goal-directed behaviors (Taylor and Jentsch 2001; Wyvell and Berridge 2000; Zhang et al. 2003) that are known to critically rely on phasic DA signaling (Tsai et al. 2009; Zweifel et al. 2009). Further, acute DREADD inhibition or activation of DA neurons decreases or increases AMPH-induced hyperlocomotor response (Runegaard et al. 2019), respectively, revealing a role for DA neuron firing activity in governing AMPH's motor effects. Importantly, in addition to modulating glutamatergic transmission via EAAT3 (Li et al. 2017; Underhill et al. 2014), AMPH has been shown to increase firing activity of DA neurons via multiple disinhibitory mechanisms, including cannabinoid receptor 1 signaling on GABA neurons (Covey et al. 2016), dopamine transporter-mediated increases in excitability (Ingram et al. 2002), and modulation of noradrenergic signaling (Shi et al. 2000). It is thus plausible that chronic DREADD-induced perturbations in firing result in the recruitment of similar homeostatic mechanisms that are also impacted by exposure to AMPH.

In contrast to our use of repeated stimulation of hM3D(Gq), a recent study using the inhibitory DREADD hM4D(Gi), reported no impact of chronic inhibition (using twice-daily injections of CNO over 16 days) of DA neurons on basal motor activity in adult mice (Salesse et al. 2020). Apart from the different methodologies used, including the use of different Cre lines and dosing regimen, hM3D(Gq) and hM4D(Gi) trigger different intracellular cascades to induce depolarization or hyperpolarization and would thus engage disparate plasticity mechanisms to shape behavior. It would be interesting to evaluate whether repeated DREADD inhibition of DA neurons impacts AMPH-induced hyperlocomotion and stereotypy, as we observed with DREADD stimulation and EAAT3 manipulation. Based on our findings, repeated chemogenetic inhibition of DA neurons might be expected to heighten response to AMPH. This idea would also be consistent with increased AMPH response that is observed following exposure to chronic stress, which also induces blunted DA firing (Antelman et al. 1980; Gomes et al. 2020; Herman et al. 1984; Moore et al. 2001).

Chronic activation of neurons typically induces plastic events and compensatory responses. In the current study, repeated DREADD activation resulted in diminished baseline firing activity of DA neurons. This is not explained by changes in sensitivity or signaling of the DREADD receptors themselves because the diminished baseline firing is observed prior to the daily administration of CNO, and the locomotor responses to each CNO dose are similar across repeated administrations. Other homeostatic mechanisms must be responsible. As one possibility, activation of muscarinic receptors can trigger release of intracellular calcium via activation of the phospholipase C cascade. A rise in NMDA receptor-independent intracellular calcium has been found to be both necessary and sufficient to trigger long-term depression (LTD) in DA neurons (Jones et al. 2000). It is thus plausible that repeated activation of DREADDs results in LTD in DA neurons with the consequences of reduced baseline neuronal firing. Both basal and AMPH responses were restored after a CNO washout period, indicating that the mechanisms responsible for homeostatic adjustment

to repeated CNO administration are temporary and dependent upon ongoing DREADD stimulation. Future studies are needed to examine the mechanisms that underlie decreased DA neuron firing following repeated DREADD stimulation, as well as restoration after a period of washout.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Conflict of interest

JV has served on advisory boards for Roche, Novartis, and SynapDx; has received research funding from NIH, Simon's Foundation, Roche, Novartis, SynapDx, Forest, Janssen, Yamo, MapLight, Seaside Therapeutics, and Acadia Pharmaceuticals; and has received editorial stipends from Wiley and Springer. The remaining authors declare no competing interests.

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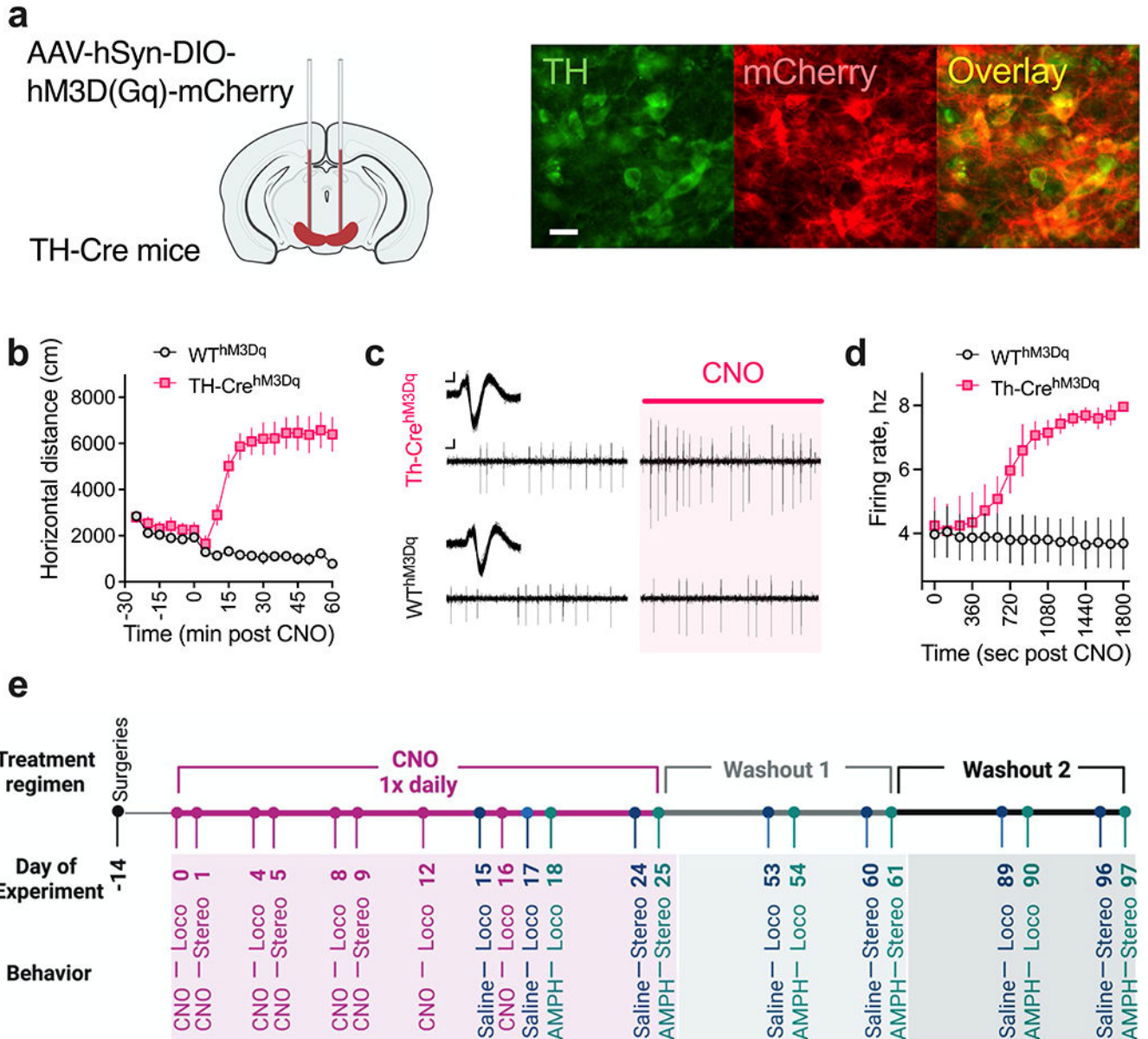
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**Fig. 1. Validation of DREADD-based activation of dopaminergic neurons.**

(a) Schematic of AAV-hSyn-DIO-HM3D(Gq)-mCherry injections into the midbrain of TH-Cre and littermate WT control mice. Shown are representative sections from the midbrain region of TH-Cre<sup>hM3Dq</sup> mice. Scale bar: 20  $\mu$ m. (b) Increased open-field locomotion in TH-Cre<sup>hM3Dq</sup> mice following acute 1 mg/kg CNO injection. N = 8 TH-Cre<sup>hM3Dq</sup> and 10 WT<sup>hM3Dq</sup> mice. (c-d) Increased firing rate of DA neurons in TH-Cre<sup>hM3Dq</sup> mice following acute 1 mg/kg CNO administration. (c) Representative extracellularly recorded DA neuronal waveforms (insets) and spike patterns from TH-Cre<sup>hM3Dq</sup> and WT<sup>hM3Dq</sup> mice before (Left) and after ~ 25 mins (Right) of CNO injection. Scale bar: Waveform, 0.05mv/0.5ms; spike patterns, 0.05mv/50ms. (d) Following a baseline recording period of 2 min, mice were administered an IP dose of 1 mg/kg CNO as recording continued for another 30 mins. N = 3 neurons/3 TH-Cre<sup>hM3Dq</sup> and 3 neurons/3 WT<sup>hM3Dq</sup> mice. (e) Experimental timeline

for longitudinal behavior. Surgeries were conducted on P60 (Day -14). After a two-week incubation period, mice were administered an IP dose of 1 mg/kg CNO once daily P74-99. CNO was administered during the test sessions on CNO test days (for locomotion: Days 0, 4, 8, 12, 16; for stereotypy: Days 1, 5, 9) and at the conclusion of the session on saline/AMPH test days (for locomotion: Days 15, 17, 18; for stereotypy: Days 24, 25). CNO was administered to mice within their home cages at ~ the same time on non-test days. Mice were tested again for saline and AMPH responses one month (Days 53-54 and 60-61) and two months (Days 89-90 and 96-97) after stopping CNO treatment.

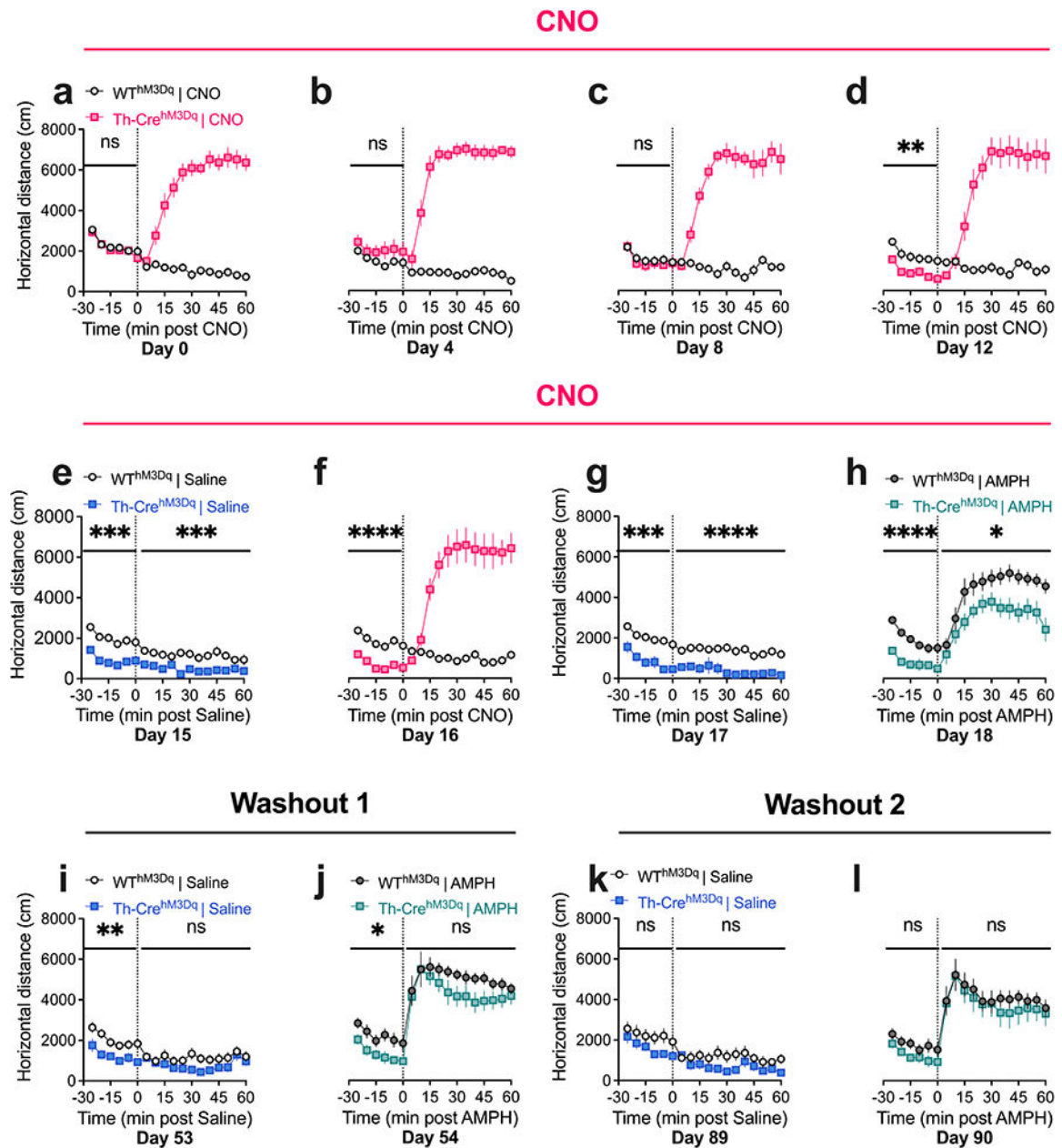
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# Locomotion

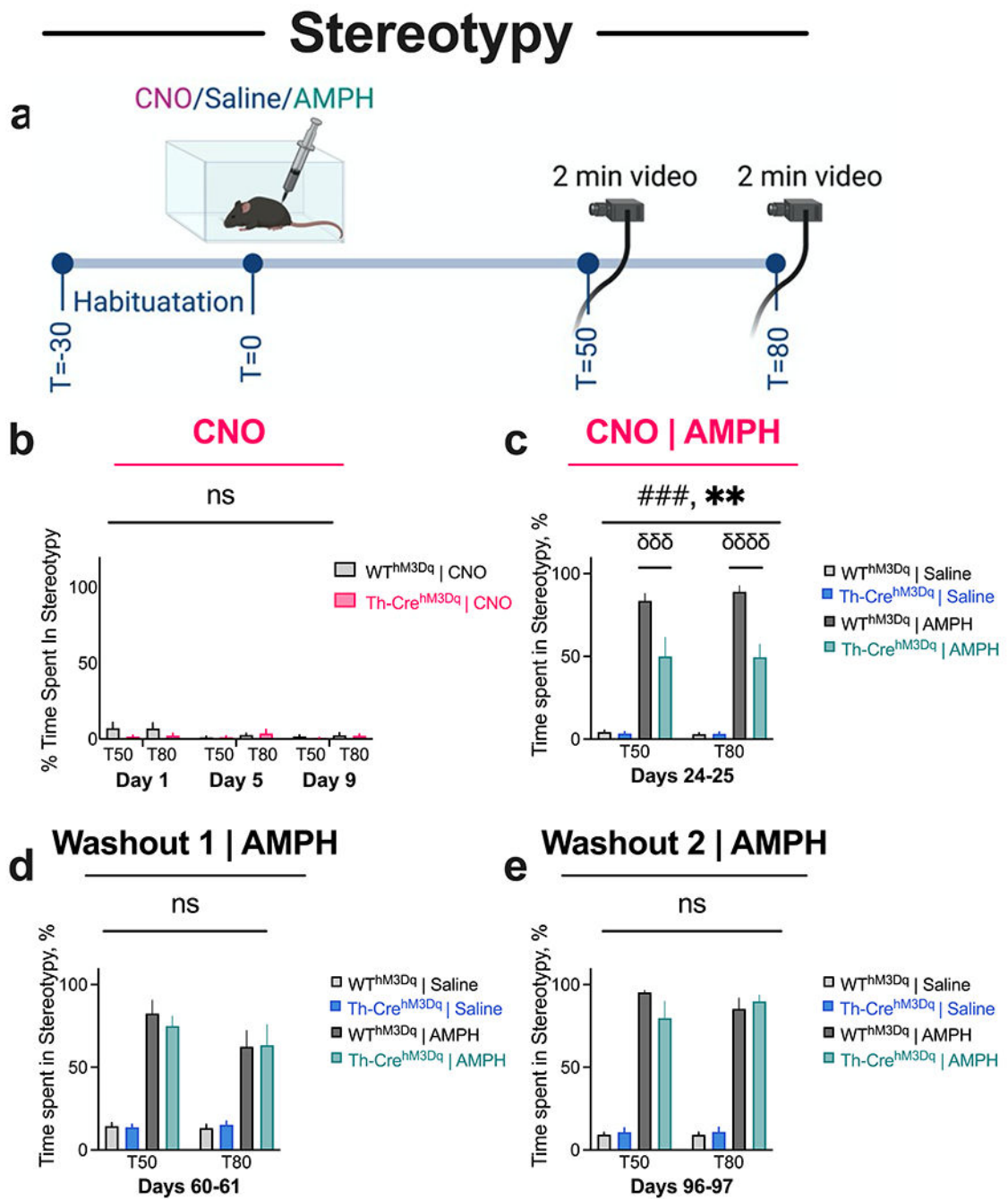


**Fig. 2. Repeated CNO administration results in diminished baseline and AMPH-induced locomotion in TH-Cre<sup>hM3Dq</sup> mice that is reversed after CNO washout.**

(a) TH-Cre<sup>hM3Dq</sup> mice display increased open-field locomotor activity following acute CNO administration. No between-genotype difference is observed in baseline locomotion on Day 0. (b-d) TH-Cre<sup>hM3Dq</sup> mice show stable increases in locomotion following repeated CNO injections. A decrease in baseline locomotion is observed in TH-Cre<sup>hM3Dq</sup> mice on Day 12. (e-h) The decreases in baseline locomotion in TH-Cre<sup>hM3Dq</sup> mice is again observed on Days 15-18 of the CNO phase. Decreases in locomotor activity is also observed after saline



administration (**e, g**). TH-Cre<sup>hM3Dq</sup> mice show diminished AMPH-induced hyperlocomotion after repeated CNO (**h**). (**i, j**) TH-Cre<sup>hM3Dq</sup> mice show diminished baseline locomotion after one month of CNO washout. No between-genotype differences are observed in AMPH-induced hyperlocomotion after one month washout. (**k, l**) TH-Cre<sup>hM3Dq</sup> mice show comparable baseline and AMPH-induced hyperlocomotor response to WT<sup>hM3Dq</sup> mice after two months of CNO washout. N = 8 TH-Cre<sup>hM3Dq</sup> and 10 WT<sup>hM3Dq</sup> mice. Main effect of genotype, \*\*\*\* $P < 0.0001$ , \*\*\* $P < 0.001$ , \*\* $P < 0.01$ , \* $P < 0.05$ ; <sup>ns</sup> $P$ , not significant. Also see Supplemental Information Fig. S11 for Area Under Curve (AUC) analysis of this dataset.



**Fig. 3. Repeated CNO administration induces decreases in AMPH-induced stereotypy in TH-Cre<sup>hM3D(Gq)</sup> mice that is rescued after CNO washout.**

(a) Timeline of Stereotypy experiment. (b) Repeated CNO treatment does not induce stereotypic behavior in TH-Cre<sup>hM3Dq</sup> and WT<sup>hM3Dq</sup> animals. (c) TH-Cre<sup>hM3Dq</sup> mice show diminished AMPH-induced stereotypy compared to WT<sup>hM3Dq</sup> mice in the CNO phase. (d-e) No between-genotype differences are observed in AMPH-induced stereotypy after 1 month (d) and 2 months (e) of CNO washout. N = 8 TH-Cre<sup>hM3Dq</sup> and 10 WT<sup>hM3Dq</sup>

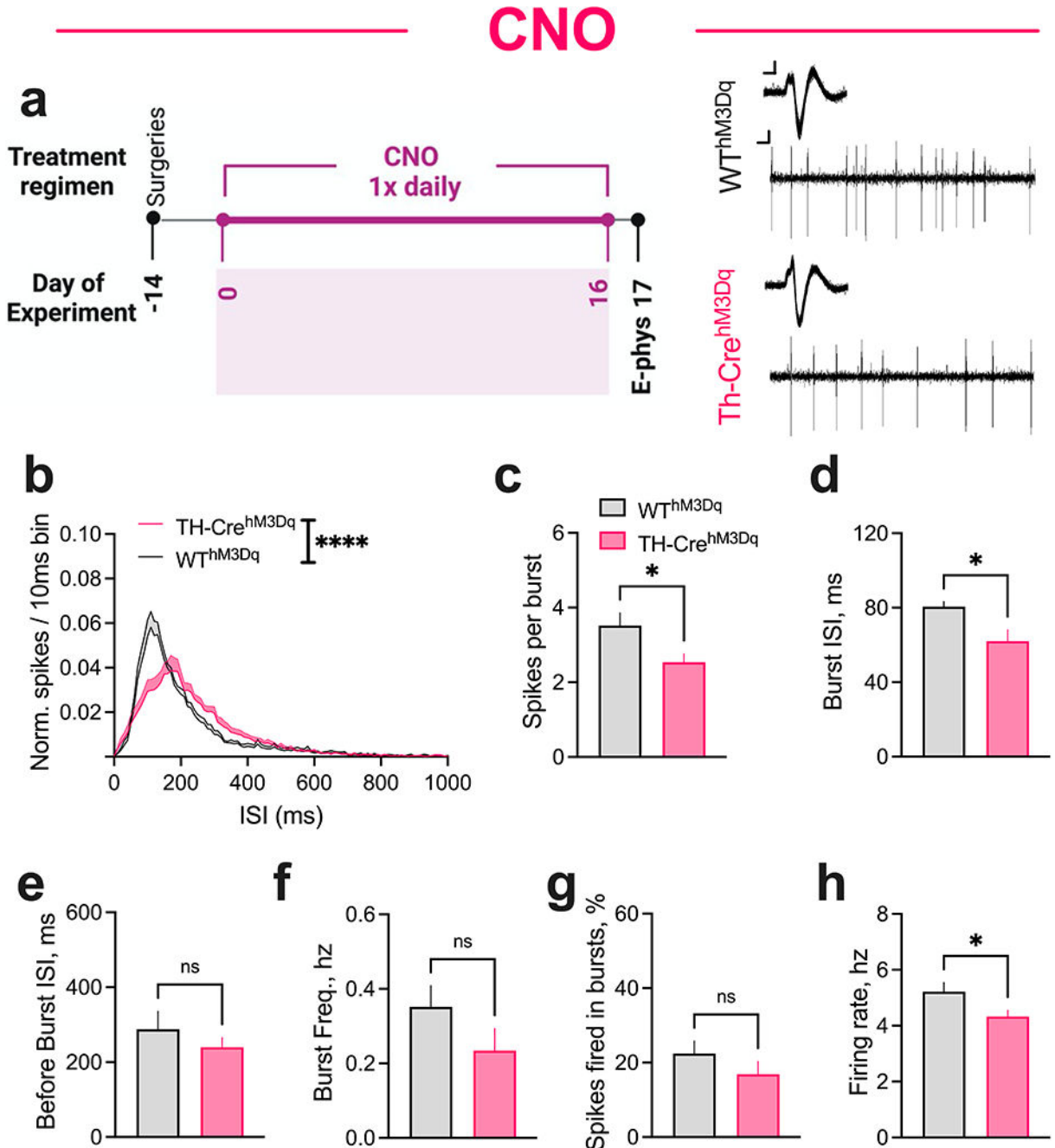
mice. Two-way interaction,  $###P < 0.001$ ; main effect of genotype,  $**P < 0.01$ ; post-hoc comparisons,  $\sigma\sigma\sigma\sigma P < 0.0001$ ,  $\sigma\sigma\sigma P < 0.001$ ;  $^{ns}P$ , not significant.

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**Fig. 4. Repeated CNO administration is associated with decreased spike firing of dopaminergic neurons in TH-Cre<sup>hM3D(Gq)</sup> mice.**

(a) CNO was administered once daily P74-90/Day 0-16. DA neurons were recorded on P91/Day 17 ~24 hours after the final CNO injection. (Left) *In vivo* recordings experimental timeline. (Right) Representative extracellularly recorded dopaminergic neuron waveforms (inset; overlay of ~100 spikes) and spike patterns from TH-Cre<sup>hM3Dq</sup> and WT<sup>hM3Dq</sup> mice. Scale bar: Waveform, 0.05 mv/0.5 ms; Spike patterns, 0.05 mv/50 ms. (b) Normalized ISI histograms from putative DA neurons showing right-shift of ISI distribution in TH-

Cre<sup>hM3Dq</sup> mice relative to WT<sup>hM3Dq</sup> control mice after 16 days of repeated CNO injections. TH-Cre<sup>hM3Dq</sup> mice display significantly decreased (c) spikes per burst and (d) average burst ISI compared to WT<sup>hM3Dq</sup> control mice. No between-genotype differences were observed in (e) average before-burst ISI, (f) burst frequency, and (g) percent spikes fired in bursts. (h) TH-Cre<sup>hM3Dq</sup> mice displayed significantly decreased firing rate compared to WT<sup>hM3Dq</sup> controls. N = 71 neurons/9 TH-Cre<sup>hM3Dq</sup> and 55 neurons/7 WT<sup>hM3Dq</sup> mice. \*\*\*\**P* < 0.0001; \**P* < 0.05; <sup>ns</sup>*P*, not significant.