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Oral application of clozapine-*N*-oxide using the micropipette-guided drug administration (MDA) method in mouse DREADD systems

Sina M. Schalbetter[1], Flavia S. Mueller[1], Joseph Scarborough[1], Juliet Richetto[1,2], Ulrike Weber-Stadlbauer[1], Urs Meyer[1,2] and Tina Notter[3]✉

The designer receptor exclusively activated by designer drugs (DREADD) system is one of the most widely used chemogenetic techniques to modulate the activity of cell populations in the brains of behaving animals. DREADDs are activated by acute or chronic administration of their ligand, clozapine-*N*-oxide (CNO). There is, however, a current lack of a non-invasive CNO administration technique that can control for drug timing and dosing without inducing substantial distress for the animals. Here, we evaluated whether the recently developed micropipette-guided drug administration (MDA) method, which has been used as a non-invasive and minimally stressful alternative to oral gavages, may be applied to administer CNO orally to activate DREADDs in a dosing- and timing-controlled manner. Unlike standard intraperitoneal injections, administration of vehicle substances via MDA did not elevate plasma levels of the major stress hormone, corticosterone, and did not attenuate exploratory activity in the open field test. At the same time, however, administration of CNO via MDA or intraperitoneally was equally efficient in activating $hM3D_{Gq}$-expressing neurons in the medial prefrontal cortex, as evident by time-dependent increases in mRNA levels of neuronal immediate early genes (*cFos, Arc* and *Zif268*) and cFos-immunoreactive neurons. Compared to vehicle given via MDA, oral administration of CNO via MDA was also found to potently increase locomotor activity in mice that express $hM3D_{Gq}$ in prefrontal neurons. Taken together, our study confirms the effectiveness of CNO given orally via MDA and provides a novel method for non-stressful, yet well controllable CNO treatments in mouse DREADD systems.

Designer receptors exclusively activated by designer drugs (DREADDs) are powerful chemogenetic tools that are widely used to manipulate and study neuronal and glial signal transduction in a brain region– and cell type–specific manner[1]. This system is based on engineered G-protein–coupled receptors that are activated by drug-like molecules such as clozapine-*N*-oxide (CNO)[2]. Cell type and brain region specificity can be obtained through intracerebral injections of adeno-associated viral constructs (AAVs), which encode DREADDs under the control of cell type–specific promoters. What makes this technique particularly useful is the fact that cellular activity can be manipulated by a single administration of the DREADD activator in freely moving animals. This has made chemogenetics one of the most widely used techniques to unravel and characterize neuronal and/or glial mechanisms regulating behavioral, cognitive and physiological functions[3].

Intraperitoneal (IP) injections are the current gold standard for administering CNO, as they allow experimenters to precisely control its dosing and timing[4]. While IP injections in rodents require the animals to be restrained[5], possible effects of manual restraints on stress responses and behavior are often ignored. Indeed, a number of studies suggest that the restraining procedure per se can increase stress-related hormones and behaviors in laboratory rodents[6,7]. Moreover, even though IP injections are generally considered to be accurate and easy to implement, their accuracy has long been questioned[8]. For example, ~20% of IP injections in rats performed by well-trained staff resulted in the substance being administered subcutaneously, retroperitoneally, into the urinary bladder or gastrointestinal tract[8]. Further consideration regarding this administration route should be made when planning chronic experiments that imply repeated CNO administrations. Repeated IP injections not only represent a chronic stressor, but they can also lead to infections at the sites of injections[9,10]. Hence, repeated IP injections of CNO can cause multiple unwanted effects, which in turn may be harmful to the animals and/or confound the experimental outcomes.

As an alternative to repeated IP injections, CNO has been provided in home-cage drinking water to activate DREADDs chronically[4,11–13]. Although this route of drug administration is non-invasive and non-stressful to the animals, it does not allow for stringent control of drug dosing and timing, both of which are indispensable for studies involving experiments that assess the acute effects of DREADD activation.

Against this background, the present study aimed to examine the suitability and effectiveness of the newly developed micropipette-guided drug administration (MDA) procedure[14] for acute CNO administration in mice. The MDA method is based on the presentation of a palatable solution consisting of sweetened condensed milk mixed with water, which motivates the animals to consume vehicle and/or drug solutions voluntarily (Fig. 1). Because of its palatable nature, mice quickly (<3 d) learn to freely drink the sweetened condensed milk solution from conventional micropipettes in the presence of the experimenter[14]. Thus, the MDA technique allows administration of substances without the need for a full restraint or invasive manipulations, thereby minimizing the stressful impact on the experimental animals. At the same time, it allows the experimenter to control for the dosing and timing of the

[1]Institute of Pharmacology and Toxicology, University of Zurich-Vetsuisse, Zurich, Switzerland. [2]Neuroscience Center Zurich, University of Zurich and ETH Zurich, Zurich, Switzerland. [3]Neuroscience and Mental Health Research Institute, Cardiff University, Cardiff, Wales, UK. ✉e-mail: nottert@cardiff.ac.uk

Fig. 1 | The three procedural steps of the MDA method in C57BL6/N mice. **a**, MDA training day 1: mice are being fully restrained and exposed to the pipette tip filled with sweetened condensed milk solution for the first time. **b**, MDA training day 2: mice are restrained solely by the tail and exposed to the sweetened condensed milk solution via a micropipette. **c**, Third day of MDA (start of actual treatment): mice voluntarily drink the sweetened condensed milk solution from the micropipette without any form of restraint.

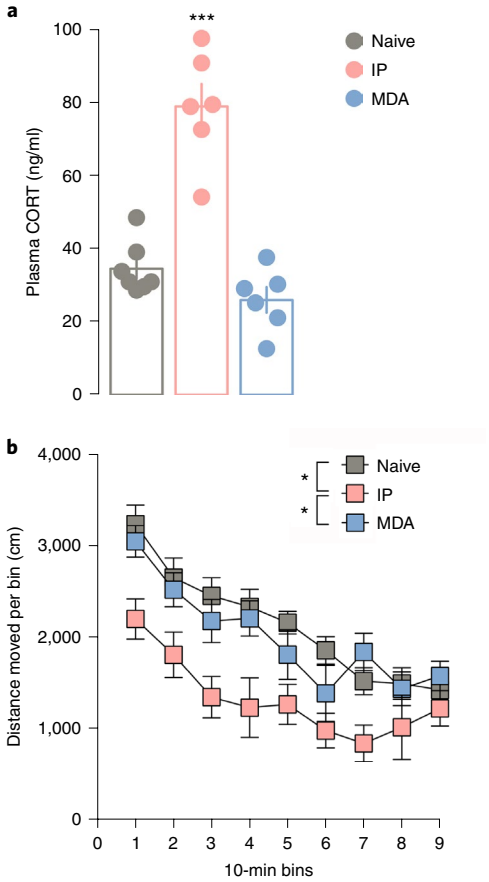


Fig. 2 | Effects of VEH administration via intraperitoneal injection or MDA on acute stress response and locomotor activity in the open field test. **a**, Plasma CORT levels 60 min after mice were treated with VEH solution by using intraperitoneal (IP) injections or the MDA method. A group of non-treated mice (Naïve) were used as a negative control. ***, $P < 0.001$, based on Tukey's post-hoc test following one-way ANOVA; n(naïve) = 7, n(IP) = 6, n(MDA) = 6. Each dot in the scatter bar plot represents an individual animal, and error bars represent s.e.m. **b**, Distance moved as a function of 10-min bins immediately after mice were treated with VEH solution by using intraperitoneal injections or the MDA method. A group of non-treated mice (naïve) were used as a negative control. *, $P < 0.05$, based on repeated-measures ANOVA; n(naïve) = 6, n(IP) = 6, n(MDA) = 6. Error bars represent s.e.m.

administered substance[14], which represents an advantage over substance administration via home-cage drinking water.

In our study, we first examined whether IP injections and MDA in mice differ with regard to inducing the stress hormone corticosterone (CORT), and in terms of affecting basal locomotor activity. We then compared the efficacy of acute CNO administration via a single IP injection or MDA to activate DREADDs. To this aim, we used a recombinant AAV that expresses the modified human muscarinic M3 G-protein–coupled (Gq) receptor under the control of the human synapsin-1 promoter ($hM3D_{Gq}V$), which was stereotactically injected into the medial prefrontal cortex (mPFC) of adult mice. Upon treatment of $hM3D_{Gq}V$-injected mice with CNO or vehicle (VEH) given via IP injection or MDA, neuronal activation was evaluated by means of measuring the mRNA expression of the immediate early genes *cFos*, *Arc* and *Zif268* 15 and 60 min after CNO administration, as well as by counting the number of cFos-positive neurons in the mPFC 120 min after CNO administration. Lastly, we assessed whether neuronal activation in the mPFC via MDA could induce locomotor hyperactivity in the open field test, an effect that has been previously described when cortical neurons—expressing $hM3D_{Gq}$ under the control of CAMKII promoter—were activated with an IP injection[15].

Results

Differential effects of IP injections and MDA on stress response and locomotor activity. We first examined whether IP injections and MDA differ with regard to inducing the major stress hormone, CORT, in the plasma of adult mice. To do so, animals were treated with VEH either via IP injection or MDA. An additional group of naïve animals, which were left undisturbed in their home cages, served as negative controls. There was a significant group effect ($F_{(2,16)} = 43.42$, $P < 0.0001$) in one-way ANOVA of plasma CORT levels 60 min after IP injection or MDA. As shown in Fig. 2a, a single IP injection significantly increased plasma CORT levels relative to MDA (Tukey's HSD test, $P < 0.0001$) or naïve controls (Tukey's HSD test, $P < 0.0001$), whereas plasma CORT levels were similar between naïve controls and animals treated with the MDA method (Tukey's HSD test, $P = 0.3456$).

We further investigated whether substance administration via IP injection or MDA may have an effect on behavioral readouts per se. To this end, we compared animals that were treated acutely with VEH via IP injection or MDA and non-treated control animals in a

standard test of locomotor activity in the open field. The analysis of distance moved revealed a significant main effect of group ($F_{(2,15)}$ = 43.42, P = 0.0132) and bins ($F_{(8,120)}$ = 45.95, P < 0.0001). As shown in Fig. 2b, a single IP injection of VEH significantly decreased locomotor activity scores compared to VEH treatment via MDA (Tukey's HSD test, P = 0.0423) or compared to non-treated animals (Tukey's HSD test, P = 0.0161). On the other hand, the locomotor activity was similar between non-treated and MDA-treated mice (Tukey's HSD test, P = 0.8749) (Fig. 2b).

Similar activation of DREADDs by CNO given via IP injection or MDA. We next examined whether CNO administered via MDA can activate DREADD-expressing neurons as efficiently as IP-injected CNO[16]. We generated mice expressing hM3D$_{Gq}$ bilaterally in the mPFC (Fig. 3a). These animals were then treated with CNO (1 mg/kg) given via IP injection (CNO/IP) or MDA (CNO/MDA) to induce neuronal activation in the mPFC. In addition, two groups of animals expressing hM3D$_{Gq}$ served as controls and were treated either with a single IP injection of VEH solution (VEH/IP) or a single dose of VEH solution administered via MDA (VEH/MDA). Neuronal activation in the mPFC was assessed by measuring the mRNA levels of the immediate early genes *cFos*, *Arc* and *Zif268* 15 and 60 min after treatment, and by quantifying cFos-positive neurons in the mPFC 120 min after treatment (Fig. 3a). Moreover, we performed an additional series of control experiments, whereby we quantified mRNA levels of the same immediate early genes (*cFos*, *Arc* and *Zif268*) in the mPFC of non-DREADD control mice (no stereotaxic injection and no hM3D$_{Gq}$ expression) after CNO or VEH treatment (given via MDA or IP injection) to rule out possible actions of CNO or the reinforced condensed milk on immediate early gene expression (Extended Data Fig. 1).

At 15 min post treatment, the mRNA levels of *Arc* and *Zif268* were significantly increased in CNO-treated compared to VEH-treated mice (Fig. 3b). The effects of CNO were independent of the route of its administration (Fig. 3b), leading to a significant main effect of treatment in the 2 × 2 ANOVA of Arc ($F_{(1,19)}$ = 4.995, P = 0.0376) and Zif268 ($F_{(1,19)}$ = 7.143, P = 0.0151). The main effect of administration route (Arc: $F_{(1,19)}$ = 0.0130, P = 0.9104; Zif268: $F_{(1,19)}$ = 2.175, P = 0.1566) and its interaction with treatment (Arc: $F_{(1,19)}$ = 0.4056, P = 0.5318; Zif268: $F_{(1,19)}$ = 1.796, P = 0.1960) were far from significant. The mRNA levels of *cFos* were not changed 15 min after CNO or VEH treatment given via IP injection or MDA (Fig. 3b).

At 60 min post treatment, the mRNA levels of all immediate early genes (*Arc*, *Zif268* and *cFos*) were significantly increased in CNO-treated compared to VEH-treated mice (Fig. 3b). Consistent with the earlier sampling interval at 15 min post treatment, the effects of CNO were independent of the route of its administration (Fig. 3c), leading to a significant main effect of treatment in the 2 × 2 ANOVA of Arc ($F_{(1,18)}$ = 88.83, P < 0.0001), Zif268 ($F_{(1,18)}$ = 167.3, P < 0.0001) and cFos ($F_{(1,18)}$ = 167.0, P < 0.0001). The main effect of administration route (Arc: $F_{(1,18)}$ = 2.706, P = 0.1173; Zif268: $F_{(1,18)}$ = 2.890, P = 0.1063; cFos: $F_{(1,18)}$ = 0.1740, P = 0.6815) and its interaction with treatment (Arc: $F_{(1,18)}$ = 0.7380, P = 0.4016; Zif268: $F_{(1,18)}$ = 1.108, P = 0.3064; cFos: $F_{(1,18)}$ = 0.1290, P = 0.7236) were not significant at the 60-min post-treatment sampling interval. In the additional control experiments (Extended Data Fig. 1), we found no significant changes in the mRNA levels of immediate early genes in the mPFC of non-DREADD control mice 60 min after treatment with CNO or VEH using the different application routes (main effect of treatment for Arc: $F_{(1,24)}$ = 0.6581, P = 0.4252; for Zif268: $F_{(1,24)}$ = 0.4176, P = 0.5243; for cFos: $F_{(1,24)}$ = 0.5284, P = 0.4743; main effect of administration route for Arc: $F_{(1,24)}$ = 1.168, P = 0.2905; for Zif268: $F_{(1,24)}$ = 0.0009, P = 0.9759; for cFos: $F_{(1,24)}$ = 1.221, P = 0.2802; two-way interaction for Arc: $F_{(1,24)}$ = 2.718, P = 0.1123; for Zif268: $F_{(1,24)}$ = 1.989, P = 0.1713; for cFos: $F_{(1,24)}$ = 2.881, P = 0.1026).

CNO treatment also led to a significant increase in the number of cFos-positive neurons in the mPFC relative to VEH-treated animals expressing hM3D$_{Gq}$ (Fig. 3d). Again, the CNO-induced increase in cFos protein levels was similar after IP injection and MDA (Fig. 3d). This led to a significant main effect of treatment in the 2 × 2 ANOVA of cFos-positive neurons ($F_{(1,18)}$ = 236.1, P < 0.0001), whereas the main effect of administration route ($F_{(1,18)}$ = 3.123, P = 0.0941) and its interaction with treatment (*cFos*: $F_{(1,18)}$ = 3.400, P = 0.0817) were not significant.

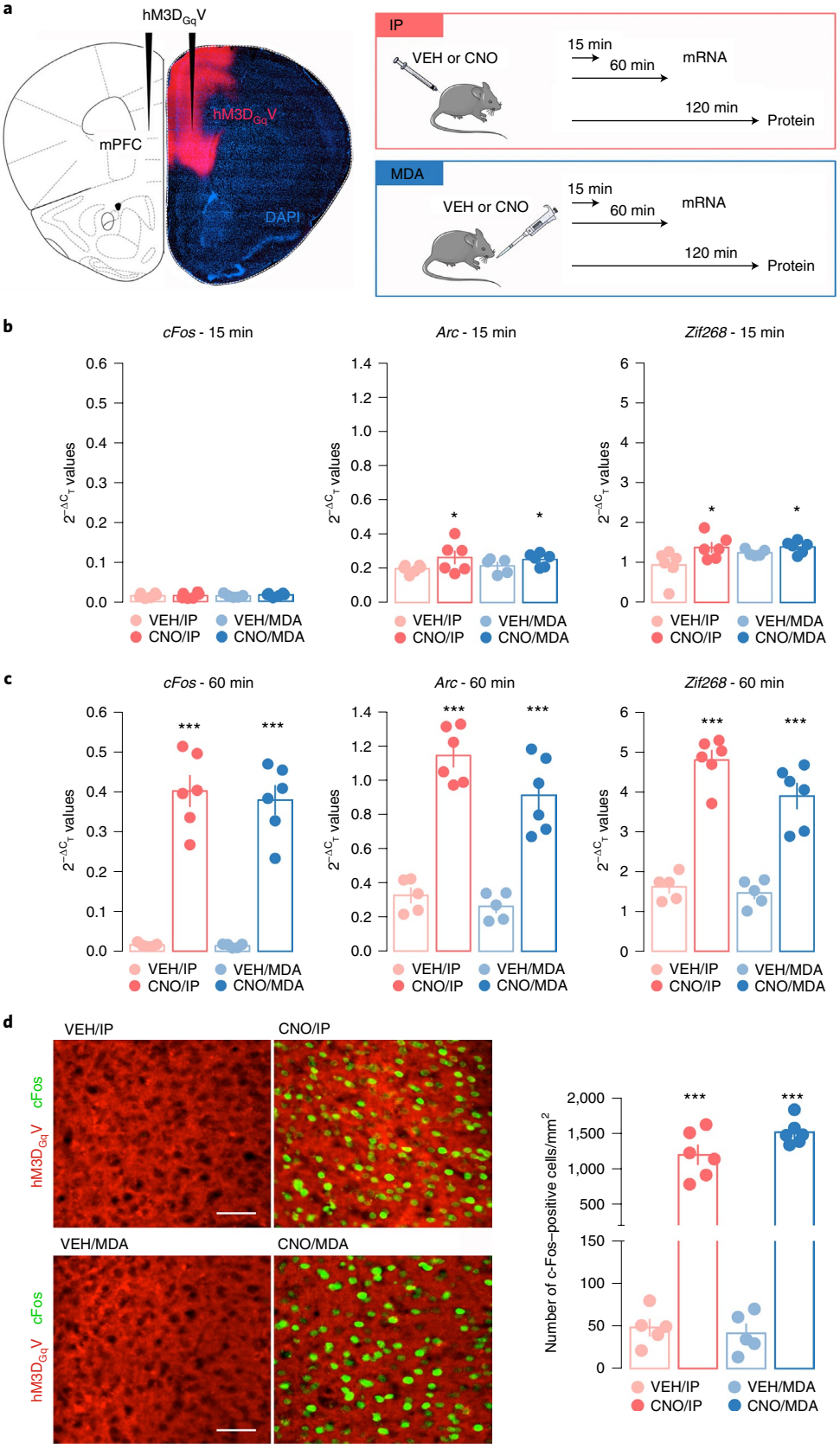
Functional verification of the effectiveness of CNO administered via MDA. We further aimed to ascertain the effectiveness of CNO administered via MDA at the functional level. To this end, we examined whether CNO administered via MDA modulates locomotor activity in mice expressing hM3D$_{Gq}$ bilaterally in the mPFC. To study the time course of the anticipated locomotor effects of CNO, mice were placed into an open field immediately after CNO (1 mg/kg) or VEH given via MDA, and the animals' distances moved were recorded for 90 min.

Figure 4a shows the locomotor activity scores expressed in bins of 5 min. Compared with VEH treatment, CNO markedly increased locomotor activity in the open field, as supported by the significant main effect of treatment in the 2 × 18 repeated measures (RM)-ANOVA ($F_{(1,11)}$ = 27.53, P = 0.0003). The CNO-induced increase in locomotor activity manifested ~40 min and persisted throughout the 90-min testing period. Additional analyses segmenting locomotor activity scores into separate 30-min bins supported this notion by revealing significant group differences at 30–60-min ($t_{(11)}$ = 3.850, P = 0.0027) and 60–90-min ($t_{(11)}$ = 2.810, P = 0.0170) intervals (Fig. 4b).

Fig. 3 | Neuronal activation in the mPFC after CNO administration via IP injection or MDA. a, Schematic illustration of the experimental approach. Mice were subjected to bilateral stereotaxic injections of recombinant AAV expressing hM3D$_{Gq}$V into the mPFC. The photomicrograph shows representative hM3D$_{Gq}$ expression in the injected mPFC, as well as the corresponding brain atlas reference region. hM3D$_{Gq}$ was activated by a single IP injection of 1 mg/kg CNO (CNO/IP) or a single oral administration of 1 mg/kg CNO using the MDA method (CNO/MDA). Two groups of animals expressing hM3D$_{Gq}$ served as controls and were treated with a single IP injection of VEH solution (VEH/IP) or a single dose of VEH solution administered via MDA (VEH/MDA). Gene expression was assessed 15 or 60 min after the CNO or VEH treatment by means of real-time PCR, whereas protein levels were measured 120 min after treatment by means of counting cFos-positive neurons in the mPFC. **b**, mRNA levels of *cFos*, *Arc* and *Zif268* in the mPFC of hM3D$_{Gq}$V-injected mice 15 min after treatment with VEH or CNO given via MDA or IP injections. *, P < 0.05, reflecting the significant main effect of treatment in 2 × 2 ANOVA; n(VEH/IP) = 6, n(CNO/IP) = 6, n(VEH/MDA) = 5, n(CNO/MDA) = 6. **c**, mRNA levels of *cFos*, *Arc* and *Zif268* in the mPFC of hM3D$_{Gq}$V-injected mice 60 min after treatment with VEH or CNO given via MDA or IP injections. ***, P < 0.001, reflecting the significant main effect of treatment in 2 × 2 ANOVA; n(VEH/IP) = 5, n(CNO/IP) = 6, n(VEH/MDA) = 5, n(CNO/MDA) = 6. **d**, The photomicrographs show representative fluorescence images of the mPFC of hM3D$_{Gq}$V-injected mice 120 min after treatment with VEH/IP, VEH/MDA, CNO/IP or CNO/MDA. Note the induction of cFos protein levels (in green) in CNO-treated relative to VEH-treated mice, which was independent of the CNO administration route. Scale bar = 50 µm. The scatter bar plot represents the number of c-Fos–positive neurons per mm^2 120 min after treatment. ***, P < 0.001, reflecting the significant main effect of treatment in 2 × 2 ANOVA; n(VEH/IP) = 5, n(CNO/IP) = 6, n(VEH/MDA) = 5, n(CNO/MDA) = 6. Each dot in the scatter bar plot represents an individual animal, and error bars represent s.e.m.

Discussion

Our study evaluated the suitability and effectiveness of the recently developed MDA procedure[14] as an alternative administration strategy for CNO in chemogenetic studies using DREADDs. The MDA method was originally established as an alternative to oral gavages in mice and takes advantage of the rodents' innate attraction



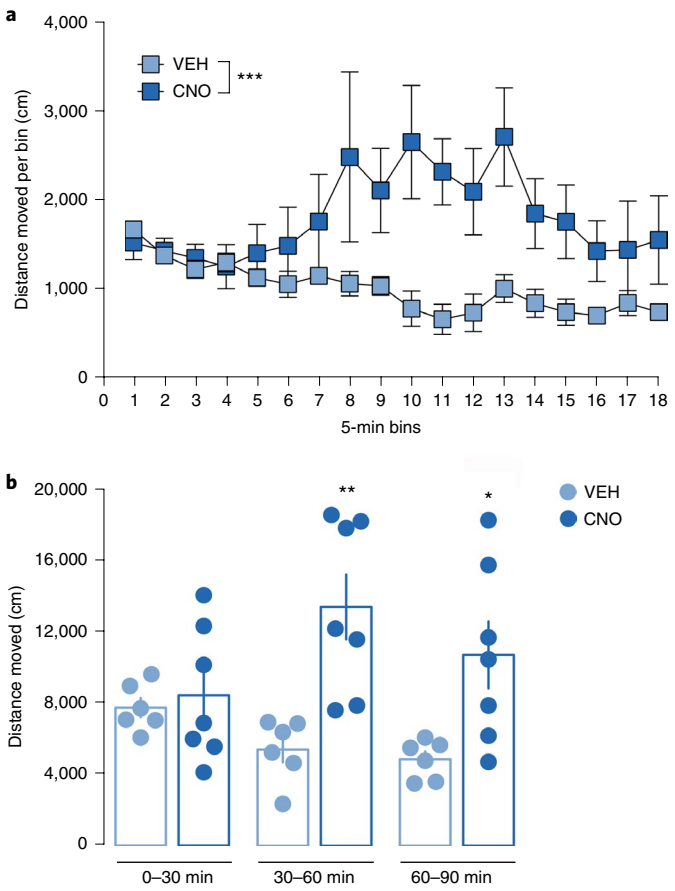


Fig. 4 | Effect of chemogenetically induced neuronal activation in the mPFC using the MDA method on spontaneous locomotor activity. **a**, Distance moved as a function of 5-min bins after VEH or CNO administration using the MDA method in mice expressing $hM3D_{Gq}$ bilaterally in the mPFC. ***, $P < 0.001$, based on RM-ANOVA; n(VEH) = 6, n(CNO) = 7. **b**, The scatter bar plots represent the total distance moved after VEH or CNO treatment during the 0–30-min, 30–60-min and 60–90-min segments of the open field test. **, $P < 0.01$, *, $P < 0.05$, based on Student's t test (two-tailed). Each dot in the scatter bar plot represents an individual animal, and error bars represent s.e.m.

toward sweet tastes[14]. The latter motivates mice to quickly learn to voluntarily drink a solution of interest from conventional micropipettes presented by the experimenter in a minimally invasive, non-stressful manner. Here, we extended the initial preclinical validation of the MDA procedure[14] to a comparison with conventional IP injections, which remain the preferred way of administering CNO (and VEH) in chemogenetic studies using DREADDs[4,17].

Consistent with previous studies[6,7], we found that IP injections of VEH in manually restrained mice elevated the major stress hormone, CORT, indicating that substance administration via IP injections is associated with a significant amount of perceived stress for laboratory animals. Furthermore, restraining the animals and injecting them with VEH solution significantly decreased locomotor activity in the open field test. These locomotor-attenuating effects were present immediately after the injection (i.e., at the beginning of locomotor testing) and persisted throughout the entire testing phase of 90 min. Although acute exposure to various stressors, including restraint stress, have generally been found to cause locomotor hyperactivity in mice[18–23], our findings of blunted locomotor activity in IP-injected mice mirror the known effects of (temporary) pain on locomotor activity in the open field test[18,19]. Even though IP injections are generally considered a relatively non-invasive administration technique, they result in puncturing the skin, muscular tissue and peritoneum, which can readily induce a temporary state of pain in IP-injected animals[24]. Importantly, unlike IP injections, administration of VEH via MDA did not change plasma CORT or locomotor behavior in comparison with non-treated mice that were left undisturbed in their home cages before testing. Thus, our findings suggest that the MDA procedure is devoid of potential confounding effects on these endocrine and behavioral parameters, both of which are key experimental readouts in preclinical research of stress-related and affective disorders[25–27].

At the same time, however, our findings demonstrate that administration of CNO via MDA is as efficient as CNO IP injections in activating neurons expressing $hM3D_{Gq}$. Indeed, compared to corresponding VEH treatment, we found that CNO administration via either route caused highly similar patterns of immediate early gene expression 15 and 60 min after treatment while having no effect in non-DREADD control mice that do not express $hM3D_{Gq}$. In line with this finding, CNO treatment resulted in a very strong increase in cFos-positive neurons in the mPFC of $hM3D_{Gq}$-expressing mice independently of its administration route. In addition, we showed that activating neurons in the mPFC via MDA-mediated CNO administration in $hM3D_{Gq}$-expressing mice resulted in increased locomotor activity relative to corresponding VEH treatment. In line with our findings, a similar pattern of locomotor hyperactivity in response to increased neuronal activity has been described previously in transgenic mice that express $hM3D_{Gq}$ under the CAMKII promoter[15]. In that study, DREADDs were activated with CNO given IP at the same dose used here (i.e., at 1 mg/kg)[15]. Although transgenic mice expressing $hM3D_{Gq}$ under the CAMKII promoter[15] differ from our experimental model system in terms of cortical $hM3D_{Gq}$ expression and neuronal activation patterns, the similarity in the onset of CNO-induced changes in locomotor activity is remarkable and generally indicates that CNO-mediated stimulation of neuronal activation is associated with locomotor hyperactivity, regardless of whether CNO is given via MDA or IP injection. Taken together, the results of our study provide molecular, cellular and behavioral evidence supporting the effectiveness of the MDA procedure in chemogenetic studies using DREADDs.

Moreover, our study suggests that the temporal onsets of CNO-induced effects are highly comparable between the MDA and IP procedures. Indeed, the induction of immediate early genes after IP- or MDA-mediated CNO treatment in $hM3D_{Gq}$-expressing mice followed comparable temporal onsets and magnitudes. Furthermore, CNO administration via IP injection[15] or MDA (present study) caused a similar onset of locomotor hyperactivity in mice expressing $hM3D_{Gq}$ under neuron-specific promoters, with hyperactivity starting to emerge ~40 min after both types of CNO administration. This similarity may appear surprising, given that an IP injection is classified as parenteral administration[10], whereas MDA is enteral. However, several studies suggest that the absorption, distribution and metabolism of IP-injected drugs rather resemble those seen after oral administration[10,28]. In fact, drugs that are injected into the peritoneal cavity are primarily absorbed by the mesenteric vessels, which then drain into the portal vein and hepatic systems[28]. Hence, the similarity in the temporal onsets of CNO-induced effects after IP or MDA administration may be explained by similar absorption, distribution and/or metabolism of the drug. The latter is the subject of intense discussion in the context of DREADD-based manipulations of central nervous system functions. For example, a recent study suggests that CNO administered systemically fails, at least under certain experimental conditions, to cross the blood–brain barrier but rather is metabolized into clozapine, which in turn may more readily enter the central nervous system[29]. Because the primary scope of our study was to evaluate the general effectiveness and suitability of MDA in DREADD systems, we did not measure

serum CNO or clozapine levels. If the observed CNO effects in $hM3D_{Gq}$-expressing mice were indeed largely attributable to converted clozapine, our data would suggest that CNO administered via MDA is as rapidly converted into clozapine as when it is administered via IP injection.

Although our study supports the use of the MDA procedure for acute chemogenetic investigations, we did not investigate the effects of repeated administration of CNO with this technique. In a recent study, however, it was found that the MDA procedure is highly suitable for administering substances of interest chronically in mice[14]. During 6-week (starting from adulthood) or 11-week (starting from weaning) treatment periods, in which mice underwent the MDA procedure daily, no dropouts of experimental subjects due to possible saturation to the condensed milk solution and/or injuries acquired during treatment were noted. Importantly, the same study further showed that chronic use of the MDA procedure did not affect body weight, suggesting that daily intake of minimal amounts of sweetened condensed milk does not affect general food intake or weight gain[14]. Together with its ease of use, low dropout rates and cost-effectiveness[14], the ability to implement the MDA procedure daily without concomitant side effects on body weight and food intake renders it highly suitable for chronic chemogenetic studies, where CNO has to be given repeatedly over a prolonged period of time. Yet, the use of the MDA procedure may be limited in experimental studies that focus on feeding behavior and/or reward processing[30,31]. Further validation is thus required to assess whether acute or chronic exposure to the (minimal amounts of) condensed milk could interfere with such physiological processes.

Although IP injections remain the gold standard in acute DREADD studies, they are less suitable for chronic studies requiring repeated injections. Repeated IP injections represent a chronic stressor and they can also lead to infections at the sites of injections[9,10]. To circumvent these potential confounders, a number of alternatives have been explored and validated recently, including implantation of minipumps[32] and intracranial cannules[33,34]. Although these techniques enable chronic CNO treatments, they are highly invasive and may affect the animals' well-being, which in turn may also confound the anticipated experimental outcomes. A less invasive alternative was recently introduced by Zhan and colleagues[4], who administered CNO through eye drops. This administration technique is intriguing in as much as it can control for both dosage and timing. Administration of CNO through eye drops, however, still involves restraint by scruffing[4], which can in turn evoke an acute stress response that could introduce a study bias[6]. By contrast, whereas the MDA procedure also involves restraint by scruffing on the first day of training, the animals quickly (<3 d) learn the procedure and are no longer required to be restrained by the time CNO is administered for experimental purposes before a test of interest. Like MDA, administering CNO through home-cage drinking water[4,11–13] is another non-invasive way to administer the drug to laboratory animals. Although this technique represents a very valuable method for certain chronic chemogenetic studies, it is not readily suited for studies that require stringent control over the dosing and timing of CNO treatment. To control timing in water-based CNO applications, a recent study has explored the alternative strategy of providing CNO in sucrose-containing drinking water during a restricted time window[4]. However, one disadvantage of this procedure is that it requires the animals to be single-housed, which in turn is well known to negatively affect multiple physiological and behavioral parameters in mice and other rodent species[35,36].

In conclusion, the present study confirms the effectiveness of CNO given orally via MDA and provides a novel method for non-stressful, yet well-controllable CNO treatments in mouse DREADD systems. It is easy to implement and cost effective and circumvents the risk of introducing injection-induced injuries to the abdominal tract or infections at the injection sites. Moreover, it improves the animals' well-being by decreasing their level of stress, distress or even pain and thus minimizes potential study bias. The full potential of this novel administration method, however, still requires further examination and extension to chronic designs.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41684-021-00723-0.

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Methods

Ethical statement. All procedures have been performed according to European Union legislation harmonized with Swiss legislation and have been approved by the Cantonal Veterinarian's Office of Zurich, Switzerland.

Animals. All experiments were performed using adult (12-week-old) male C57Bl6/N mice (Charles Rivers). They were group-housed (four to five animals per cage) in individually ventilated cages (Allentown Inc.) as previously described[37]. The cages were kept in a specific-pathogen-free holding room, which was temperature- and humidity-controlled (21 ± 3 °C, 50 ± 10%) under a reversed light–dark cycle (lights off: 09:00 AM–09:00 PM). All animals had ad libitum access to standard rodent chow (Kliba 3336) and water throughout the entire study. All procedures were conducted during the dark cycle. All efforts were made to minimize the number of animals used and their suffering. A list of the number of animals used in the different experiments is provided in Table 1.

DREADD system. The following AAV wase used: AAV8-hSyn1-hM3D(Gq)-mCherry (hM3DGqV). It was purchased from the Viral Vector Facility of the University of Zurich, Switzerland (www.vvf.uzh.ch) and was injected bilaterally in the mPFC (anteroposterior = +2.0 mm, mediolateral = +0.3 mm, dorsoventral = −2.0 mm, with reference to bregma) by using stereotaxic surgery (see below).

Stereotaxic surgery. Stereotaxic surgery was performed as previously described[16]. In brief, the mice were anesthetized by inhalation of 2.5–3.0% isoflurane (ZDG9623V; Baxter) in oxygen. Once fully anesthetized, they were injected with the analgesic Temgesic (buprenorphine, 0.1 mg/kg, s.c.; Reckitt Benckiser) and placed into the stereotaxic frame (MTM-3; World Precision Instruments) while kept under constant isoflurane/oxygen flow (1–3% isoflurane in 200 ml of oxygen/min). All mice were kept on a heating pad (ATC1000; World Precision Instruments) to control their body temperature during the entire surgery. They received a bilateral injection of 600 nl of AAV at a flow rate of 10 nl/s into the mPFC (anteroposterior = +2.0 mm, mediolateral = +0.3 mm, dorsoventral = −2.0 mm, with reference to bregma) by using a NanoFil needle and syringe (NanoFil, NF35BV; World Precision Instruments) connected to an automated pump system (UMP3T-1; World Precision Instruments). After injection of the AAV, the needle was kept in place for 5 min to avoid reflux of the virus and then retracted. The incision was sutured with a surgical thread (G0932078; B. Braun). For recovery, the mice were initially placed into a temperature-controlled chamber (Harvard Apparatus), after which they were placed back into their home cage and closely monitored for three consecutive days.

MDA procedure. The MDA procedure was based on the use of a palatable solution in the form of sweetened condensed milk (Migros Kondensmilch; Migros) mixed with Milli-Q water. The condensed milk contained unskimmed cow's milk (59%), sugar (55 g per 100 g) and stabilizer E339. It was diluted with water to yield an 80% milk stock solution (mixed on a heated stirrer to get a homogeneous solution, which can be stored for ≤2 d at 4 °C). On the day of administration, the stock solution was further diluted with either VEH solution (0.9% NaCl; B. Braun) or a 1 mg/ml CNO (Enzo Life Sciences) dissolved in 0.9% NaCl stock solution in a 1:1 ratio yielding a final milk working solution of 40%. VEH and CNO solutions were administered by using a volume of 2 ml/kg and were provided with a conventional single-channel p200 micropipette (Gilson Pipetteman, Thermo Fisher Scientific).

As previously described[14], all animals were first trained to drink the condensed milk solution from the micropipette on two consecutive days, with one training session per day. In brief, on the first day of training, the mice were restrained by scruffing and exposed to the milk solution, by offering the pipette tip containing the VEH solution to the mouth until the mouse began to lick the pipette tip. As soon as the mouse began to lick the tip, a small pressure to expel the VEH solution from the pipette was applied, allowing the mouse to drink the entire solution (Fig. 1a). On the second day of training, a looser restraint was used, such that the mice were restrained solely by the tail on the metal grid of the food hopper (Fig. 1b). Again, the pipette was continually positioned next to the mice's mouth until they started to lick and drink the content. On the third day, by the time the treatment/experiment began, the mice required minimal/no restraint for them to voluntarily drink the working solution (Fig. 1c). Delivery was considered complete when the mouse drank the entire content of the pipette. To avoid loss of drug in facial hair, solutions were expelled from the pipette tip only when the mouse was actively licking. For MDA training and subsequent implementation, the time commitment was generally found to be low. Indeed, a typical MDA session did not exceed 1 min per mouse, including taking the mouse out of the cage, restraining the mouse (on days 1 and 2 of the MDA training) and having the mouse drink the condensed milk via MDA. Thus, MDA training can be implemented as a part of the typical handling habituation before behavioral testing.

Activation of DREADDs. After a 2-week recovery period from the stereotaxic surgery, the animals were treated with CNO (1 mg/kg; Enzo Life Sciences) dissolved in 0.9% NaCl (B. Braun) or with 0.9% NaCl VEH solution only. The dose of CNO (1 mg/kg) was chosen on the basis of previous chemogenetic studies in rodents[1,15,16,38,39]. All substances were injected IP by using an injection volume of 5 ml/kg or provided via the MDA procedure (see above) by using an administration volume of 2 ml/kg. All animals that were subjected to an IP injection were habituated to handling and restraint by scruffing on two consecutive days before the actual IP injection. CNO solutions were protected from light during all procedures. The animals were placed back into their home cages and sacrificed 15 or 60 min (gene expression analyses) or 120 min (protein expression analyses) after VEH or CNO administration, or they were immediately placed into the open field to measure locomotor activity (see below).

Table 1 | Number of mice used in each experimental group

Experiment	VEH/IP	CNO/IP	VEH/MDA	CNO/MDA	Naïve
Real-time PCR (15 min)	6	6	5	6	–
Real-time PCR (60 min)	5	6	5	6	–
Control real-time PCR (60 min)	7	7	7	7	–
Immunohistochemistry	5	6	5	6	–
Locomotor activity (treatment)	–	–	6	7	–
Locomotor activity (treatment type)	6	–	6	–	6
Plasma-CORT	6	–	6	–	7

'Naïve' refers to animals that remained undisturbed in their home cage. 'Control' refers to control experiments, in which C57Bl6/N mice were used that did not express hM3DGq (non-DREADD control mice).

Quantitative real-time PCR. RNA extraction and quantitative real-time PCR analyses were performed according to previously established protocols[16,40,41]. In brief, the animals were deeply anesthetized with an overdose of Esconarkon ad us. vet. (Streuli Pharma AG) and transcardially perfused with 20ml of ice-cold, artificial cerebrospinal fluid (pH 7.4)[42]. After decapitation, the brains were immediately extracted from the skull, frozen on dry ice and stored at −80 °C until further processing. The brains were then cut into 1-mm coronal brain sections using razorblade cuts, and subsequent micro-dissection of the mPFC (bregma: +2.0 to +1.5 mm) was performed. Total RNA was isolated by using the SPLIT RNA extraction kit (008.48; Lexogen). The procedure was conducted according to the manufacturer's instructions, and the resulting RNA was quantified by Nanodrop (DeNovix DS-11+ spectrophotometer; Labgene Scientific SA). Quantitative real-time PCR was conducted with iScript one-step real-time PCR kits and a Taqman real-time system (CFX384; Bio-Rad Laboratories) as previously described[16,40,41]. The levels of *cFos, Arc* and *Zif268* mRNA were quantified by using custom-made primers purchased from Eurofins Genomics GmbH (Table 2). Ribosomal phosphoprotein (36B4; Table 2) was used as the housekeeper control, as validated previously[16,40,41]. Relative gene expression was calculated with the $2^{-\Delta\Delta CT}$ method[43].

Locomotor activity in the open field. Locomotor activity was measured by using a standard open field exploration task[44]. The apparatus consisted of four identical open field arenas (40 × 40 × 35 cm high) made of white polyvinyl chloride. It was positioned in a testing room with diffused lighting (~30 Lux in the center of the arena). A digital camera was mounted above the arena, captured images at a rate of 5 Hz and transmitted them to a PC running the EthoVision (Noldus Technology) tracking system. Immediately after treatment, animals were placed into the open fields, and their locomotor activities were recorded for 90 min.

Immunohistochemistry and fluorescence microscopy. Immunofluorescence staining was performed according to previously established protocols[16,45]. Two hours after treatment the animals were perfused trancardially with 20 ml of ice-cold artificial cerebrospinal fluid (pH 7.4), followed by 18h post-fixation in 4% phosphate-buffered paraformaldehyde and cryoprotection in 30% sucrose solution in PBS[16,42,45]. The brain samples were cut coronally with a sliding microtome at 30 µm (series of eight) and stored at −20 °C in cryoprotectant solution (50 mM sodium phosphate buffer (pH 7.4) containing 15% glucose and 30% ethylene glycol; Sigma-Aldrich) until further processing. For c-Fos staining, the slices were rinsed with Tris buffer (pH 7.4) before incubation with the c-Fos primary antibody (Santa Cruz Biotechnology cat. no. sc-52; RRID: AB_2106783; dilution 1:1,000). The primary antibody was diluted in Tris buffer containing 0.2% Triton X-100 and 2% normal serum, and the sections were incubated free-floating under constant agitation (100 rpm) overnight at 4 °C. The sections were then washed three times for 10 min in Tris buffer, incubated for 30 min at room temperature with the secondary antibody coupled to Alexa488 (diluted 1:1,000; Molecular Probes). After incubation, which was shielded from light, the sections were washed

Table 2 | List of custom-designed probe and primer sequences used for the real-time PCR of various genes of interest and reference gene (36B4)

Gene	Forward primer	Reverse primer	Probe
c-Fos	5′-TCCTTACGGACTCCCCAC-3′	5′-CTCCGTTTCTCTTCCTCTTCAG-3′	5′-TGCTCTACTTTGCCCCTTCTGCC-3′
Arc	5′-TGCAGATTGGTAAGTTGCCGA-3′	5′-TGTGCAACCCTTTCAGCTCT-3′	5′-tTCTGTTGACCGAAGTGTCCA-3′
Zif286	5′- AGCGCCTTCAATCCTCAAG-3′	5′- TTTGGCTGGGATAACTCGTC-3′	5′- CAACCCTATGAGCACCTGACCACA-3′
36B4	5′-AGATGCAGCAGATCCGCAT-3′	5′-GTTCTTGCCCATCAGCACC-3′	5′-CGCTCCGAGGGAAGGCCG-3′

thoroughly three times for 10 min in Tris buffer, mounted onto gelatinized glass slides, coverslipped with Dako fluorescence mounting medium and stored in the dark at 4 °C. Data collection was performed with a Zeiss AxioObserver Z1 inverted widefield microscope using a 20× (air, numerical aperture 0.4) objective. For each animal, one image per hemisphere was acquired from four consecutive sections containing the prelimbic and infralimbic area of the mPFC (bregma: +2.0 to +1.5 mm). Hence, a total of eight images were analyzed per animal. Cell counting was performed with the use of ImageJ software by an experimenter blinded to the treatment conditions in the form of coding by numbers. For image analysis, background subtraction and a threshold were applied to the images so that an optimal representation of c-Fos–positive neurons was achieved. Cells that clustered together were separated by the watershed function. The number of cells was then counted by the 'analyze particle' function and presented as the number of cells per square millimeter.

Quantification of plasma CORT. Plasma CORT levels were measured 60min after treatment with VEH solution given via IP injection or MDA. Mice that were assigned to the MDA procedure underwent habituation to the treatment method on two consecutive days before the test day (see above). Animals assigned to IP injections were handled by the experimenter on two consecutive days before the test day. One group of animals was kept undisturbed and used as the baseline. CORT was then measured after treatment on the third day as described before[14,37]. In brief, blood was collected through cardiac puncture in mice deeply anesthetized with an overdose of Esconarkon ad us. vet. (Streuli Pharma AG). Mice were killed between 10:00 AM and 1:00 PM to eliminate any effect on plasma CORT levels due to the circadian rhythm. After collection, the blood samples were spun at 4 °C for 10 min to separate the plasma, and CORT was analyzed with the DetectX corticosterone enzyme immunoassay kit (Arbor Assays) according to the manufacturer's instructions.

Statistical analyses. All data met the assumptions of normal distribution and equality of variance and were analyzed by using parametric ANOVA or independent Student's *t* test (two-tailed). All real-time PCR and immunofluorescence data were analyzed by using a 2 × 2 (administration route × drug) ANOVA, whereas locomotor activity in the open field was analyzed by using a 3 × 9 (group × 10-min bins) and a 2 × 18 (administration route × 5-min bins) RM-ANOVA. Plasma CORT levels were analyzed by using a one-way ANOVA. Whenever appropriate, ANOVAs were followed by Tukey's post-hoc test for multiple comparisons. All statistical analyses were performed by using SPSS Statistics (version 22.0; IBM) and Prism (version 7.0; GraphPad Software). Statistical significance was set at $P < 0.05$.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author Contributions

S.M.S. designed and performed research, analyzed data and contributed to the preparation of the manuscript. F.S.M., J.S., J.R. and U.W.-S. performed research. U.M. designed research and contributed to the preparation of the manuscript. T.N. designed and performed research, analyzed data and wrote the manuscript.

Competing interests

Unrelated to the present study, U.M. has received financial support from Boehringer Ingelheim Pharma GmbH & Co. and from Wren Therapeutics Ltd. All authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to T.N.

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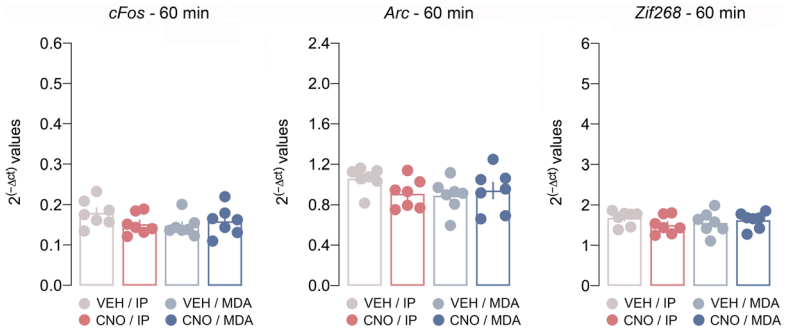

Extended Data Fig. 1 | Immediate early gene expression in the mPFC after CNO (1 mg/kg) or VEH administration via IP injection or MDA in the absence of the modified human muscarinic M3 G-protein-coupled receptor (DREADD). The scatter bar blots represent mRNA levels of *cFos*, *Arc* and *Zif268* in the mPFC of adult (12-week-old) C57Bl6/N mice 60 min after treatment with VEH or CNO given via MDA or IP injections; *n*(VEH/IP) = 7, *n*(CNO/IP) = 7, *n*(VEH/MDA) = 7, *n*(CNO/MDA) = 7. Each dot in the scatter bar plot represents an individual animal, and error bars represent s.e.m.

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Software and code

Policy information about availability of computer code

Data collection	Behavioural data were collected with a digital camera, which was mounted above the arena that captured images at a rate of 5 Hz and transmitted them to a PC running the EthoVision (Noldus Technology, Wageningen, The Netherlands) tracking system. qPCR data collection: RNA was quantified by Nanodrop (DeNovix DS-11+ Spectrophotometer, Labgene Scientific SA, Switzerland). Quantitative real-time PCR was conducted with iScript one-step RT-PCR kits and a Taqman real-time system (CFX384, Bio-Rad Laboratories, Cressier, Switzerland). The data were exported to and further analysed with Microsoft Excel (version 16.44). Immunohistochemistry data collection was performed with a Zeiss AxioObserver Z1 inverted widefield microscope using a 20× (air, NA 0.4) objective. Cell counting was performed using the ImageJ software and Microsoft Excel (version 16.44). Plasma Cortisosterone data collection was performed with the DetectX® Corticosterone Enzyme Immunoassay kit (Arbor Assays, Ann Arbor, USA) and Microsoft Excel (version 16.44).
Data analysis	Data analyses were performed using SPSS Statistics (version 22.0, IBM, Armonk, NY, USA) and Prism (version 7.0; GraphPad Software, La Jolla, CA, USA).

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	Based on the author's previous findings using DREADD-based murine models (Notter et al., 2020 Molecular Psychiatry) a minimum sample size of 5 was chosen.
Data exclusions	No data was excluded.
Replication	There were no additional experimental cohorts used to replicate the finidngs described in this study. In a first pilot study we found that the number of c-Fos positive cells were increased after animals were exposed to CNO via MDA or IP, an effect that is consistent with the data reported here.
Randomization	Adult male mice that underwent stereotaxic injection, and animals that were left undisturbed in their homecage (naive controls), were ramdomly allocated to the different treatments.
Blinding	All data collection and analyses were performed by an experimenter who was blinded to the experimental conditions. Blinding was achieved by coding. Unblinding was performed only once all data were collected.

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Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology
☐	☒ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	Rabbit c-Fos primary antibody, Santa Cruz Biotechnology, Catalogue number: sc-52, Clone ID: 4, Lot Number: 10309. RRID:AB_2106783.
Validation	Relevant citations for the validation of this antibody can be found here: https://antibodyregistry.org/search.php?q=AB_2106783.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals	Adult (12 weeks old) male C57Bl6/N mice (Charles Rivers, Sulzfeld, Germany)
Wild animals	The study did not involve wild animals.
Field-collected samples	The study did not involve sample collection in the field.
Ethics oversight	All procedures have been approved by the Cantonal Veterinarian's Office of Zurich, Switzerland (Permit Number: Permit ZH196/16).

Note that full information on the approval of the study protocol must also be provided in the manuscript.