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***In vivo* electrophysiological validation of DREADD-based modulation of pallidal neurons in the non-human primate.**

Marc Deffains^{1, 2, #}, *Tho Hai Nguyen*^{1, 2}, *Hugues Orignac*^{1, 2}, *Nathalie Biendon*^{1, 2}, *Sandra Dovero*^{1, 2}, *Erwan Bezard*^{1, 2} & *Thomas Boraud*^{1, 2, 3}

1 Université de Bordeaux, Institut des Maladies Neurodégénératives (IMN), UMR 5293, 33076 Bordeaux, France

2 Centre National de la Recherche Scientifique, IMN, UMR 5293, 33076 Bordeaux, France

3 IMN Clinique, Hôpital Pellegrin, Centre hospitalier Universitaire de Bordeaux, Place Amélie Raba Léon, 33076 Bordeaux, France

Correspondence: marc.deffains@u-bordeaux.fr

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Abstract

Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) are widely used in rodents to manipulate neuronal activity and establish causal links between structure and function. Their utilization in non-human primates (NHPs) is however limited and their efficacy still debated. Here, we recorded and examined the neuronal activity in the hM4Di DREADD-transduced and hM4Di DREADD-free GPe of two anesthetized animals following local intra-GPe microinjection of clozapine-N-oxide (CNO). Our results revealed that the neuronal activity of the well-isolated units recorded in the hM4Di DREADD-transduced GPe exhibited diverse patterns in timing and polarity (increase/decrease) of firing rate modulations following CNO injection. Nevertheless, significant decreases in activity were more frequent (and more pronounced) than significant increases in activity during CNO injection (6/18 vs. 3/18 units), and were exclusive after CNO Injection (8/18 units). In contrast, only one of the 8 well-isolated units recorded in hM4Di DREADD-free GPe exhibited a significant increase in activity after CNO injection. Overall, the number of units exhibiting a significant period-related decrease following CNO injection were significantly larger in hM4Di DREADD-transduced GPe than in the hM4Di DREADD-free GPe 8/18 [(44.4%) vs. 0/8 (0%)]. Moreover, post-mortem histochemical analysis revealed that hM4Di DREADDs were expressed at high level in the GPe neurons located in the vicinity of the viral vector injection sites. Our results therefore show *in vivo* hM4Di DREADD-based inhibition of pallidal neurons in the NHP model and reinforce the view that DREADD technology can be effective in NHPs.

Introduction

Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) technique can be used as a powerful tool to remotely, reversibly and repeatedly manipulate the neural mechanisms underlying higher brain functions with minimal invasiveness and maximal control of anatomical coverage (Roth, 2016). The hM4Di receptor (i.e., engineered version of the M4 muscarinic acetylcholine receptor) is a popular muscarinic-based DREADD that can reduce the activity of DREADD-transduced neurons when activated by clozapine-n-oxide (CNO). This DREADD technology is widely used in rodents (e.g., Ferguson *et al.*, 2011, 2013; Chang *et al.*, 2015; Smith *et al.*, 2016); however, its efficacy in non-human primates (NHPs) is still debated. To date, only few NHP studies using DREADDs have been conducted (Eldridge *et al.*, 2016; Grayson *et al.*, 2016; Nagai *et al.*, 2016; Upright *et al.*, 2018; Bonaventura *et al.*, 2019; Galvan *et al.*, 2019; Raper *et al.*, 2019). Moreover, although some of them have already observed DREADD-based behavioral effects (Eldridge *et al.*, 2016; Grayson *et al.*, 2016; Upright *et al.*, 2018; Raper *et al.*, 2019), there is still no direct evidence that neuronal activity can be manipulated in monkeys using DREADDs and very little attention has been paid to the basal ganglia (BG).

BG are a set of interconnected sub-cortical structures involved in a broad diversity of brain functions (e.g., learning, action selection and decision-making) and their dysfunction leads to common neurological and psychiatric disorders. Therefore (and given the unique value of the NHP model in translational research), the validation of the DREADD technology in the NHP BG network is of utmost importance to determine the neural mechanisms underlying the BG functions and related-disorders. Today, GPe is regarded as the central structure of the BG network (Goldberg & Bergman, 2011; Deffains *et al.*, 2016). Indeed, GPe is reciprocally connected to the striatum and the subthalamic nucleus (i.e., the BG input structures), and is a major source of innervation of the BG output structures (i.e., the internal segment of the globus pallidus and the substantia nigra pars reticulata) (Parent & Hazrati, 1995). GPe is therefore crucial in the orchestration of BG activity and in BG information processing. For all these reasons, we tested DREADD expression in the NHP GPe and validated DREADD-based modulation of GPe neurons using electrophysiological recordings in the anesthetized monkey. To do so, we transduced NHP GPe neurons with hM4Di receptor and recorded single-unit activity in the hM4Di-DREADD-transduced and hM4Di-DREADD-free GPe following local intra-GPe microinjections of CNO.

Materials and Methods

Animals

Two rhesus monkeys (F and E, *Macaca mulatta*) weighing ~ 7-8kg, were used in this study. The monkeys were housed in the animal facility of the Institute of Neurodegenerative Diseases (UMR CNRS 5293, Bordeaux, France) under standard conditions (12/12h day/night cycle, ~ 60% humidity and ~ 22°C). All experimental protocols comply with Council Directive of 2010 (2010/63/EU) of the European Community and the National Institute of Health Guide for the Care and Use of Laboratory Animals. The proposed research has received agreement from French Ethical Committee for Animal Research CE50 (agreement number: C33063268).

Viral vectors and surgical procedures

To transduce GPe neurons with hM4Di DREADDs, we used adeno-associated virus (AAV) expressing hM4Di receptor fused to mCherry fluorescent protein tag under human synapsin promoter [AAV2-hSyn-hM4D(Gi)-mCherry (3×10^{12} and 1.1×10^{13} viral particles/ml for monkeys F and E, respectively), Addgene, Cambridge, MA, USA]. The plasmid was provided to Addgene by Dr. Bryan Roth (UNC).

Surgeries were performed under aseptic conditions and isoflurane and O₂ deep anesthesia, following induction with ketamine (10mg/kg IM) and atropine (0.05mg/kg IM). Body temperature, heart rate blood pressure and SpO₂ were monitored throughout all surgical

procedures. During surgery, the head of the animal was fixed in a stereotaxic frame (M2e-Unimécanique, Eaubonne, Val-d'Oise, France) and craniotomies were made above GPe (left and right GPe in monkeys F and E, respectively). Viral vectors were administered in 4 injections within the GPe using a UMP3 microsyringe pump injector (World Precision Instruments, Sarasota, FL, USA) with 100 μ l Hamilton syringe. For each injection, a needle was lowered to the injection site and 20 μ l of the viral vector was infused at a rate of 2 μ l/min. The NHP GPe being much bigger than the GPe of rats [volume of the GPe of the macaque is ~25-fold larger than volume of rat GPe (Hardman *et al.*, 2002)], we chose to inject a relatively large volume of viral vector (4*20 μ l per GPe) to maximize our chances to find and record hM4Di DREADD-transduced GPe neurons. Injection sites within GPe were ascertained according to the stereotaxic coordinates of GPe (Paxinos *et al.*, 2000) and guided by ventriculography and single-unit electrophysiological recordings. A ventriculographic cannula was introduced into the anterior horn of the lateral ventricle and a contrast medium (Omnipaque) was injected (Piron *et al.*, 2016). Corrections in the position of the injection sites were performed according to the line between the anterior and posterior commissures. Moreover, before the needle was lowered to the injection site, neuronal activity (see *Electrophysiology and intracerebral injections* for details) was recorded at the level of the viral vector injection sites to guarantee that the recorded activity corresponds to the well-known activity of the GPe. Extracellular recording methods in NHPs allow us to characterize two distinct populations of GPe neurons based on their discharge features (DeLong, 1971; Elias *et al.*, 2007; Bugaysen *et al.*, 2010): high-frequency discharge interrupted by pauses vs. low-frequency discharge with occasional brief high frequency bursts.

Theoretical coordinates of the injection sites (in mm) from the vertical plane passing through the interaural line (AP), the midline (L) and the horizontal plane passing through the interaural line (D) were: AP:18.3, L:5, D:16; AP:18.3, L:7, D:16; AP:16.5, L:6, D:16; AP:16.5, L:9, D:16 (Paxinos *et al.*, 2000). After each injection, there was a 5-min waiting period to minimize viral vector spread during retractions. Analgesics (Metacam, 0.2mg/kg IM) were administered during the first 3 postoperative days. A 4-week minimum waiting period for allowing hM4Di DREADD expression was allotted before starting recordings.

Drugs

CNO (Tocris) was dissolved and diluted with 0.9% saline to produce a final concentration of 1mM. Aliquots were stored at -20°C and protected from light until further processing. Although we did not assess animal behavior (anesthetized animals), CNO concentration was selected based on several studies in rodents that reported behavioral effectiveness (with no DREADD-independent behavioral effects) of *in vivo* 1mM CNO microinjection in ventral

tegmental area (Mahler *et al.*, 2014), lateral septum (McGlinchey & Aston-Jones, 2018), dorsal hippocampus (Ge *et al.*, 2017), or orbital cortex (Lichtenberg *et al.*, 2017).

Electrophysiology and intracerebral injections

During the recording sessions, the animal was re-anesthetized (see *viral vectors and surgical procedures* for the details) and its head was re-immobilized in the stereotaxic frame (M2e-Unimécanique, Eaubonne, Val-d'Oise, France). Narylene-coated tungsten microelectrodes (impedance at 1 kHz: ~1 M Ω) were manually advanced within the GPe to the viral vector injection sites. The electrical activity was amplified by 100, band-pass filtered from 250 to 6000Hz using a hardware two-pole Butterworth filter and sampled at 25kHz (advanced wireless W2100, Multichannel Systems, Reutlingen, BW, Germany). After radiographic and electrophysiological confirmation of the electrode position, 25 μ l Hamilton syringe [mounted on a UMP3 microsyringe pump injector (World Precision Instruments, Sarasota, FL, USA)] was filled with CNO (Bio-Techne, Minneapolis, MN, USA) and needle was lowered to its final position (Fig.1A, B and C). When a neuron was found within ~2mm radius from the tip of the needle, its activity was recorded during 10-min baseline period in order to assess the stability of its activity (before starting CNO injection). If its activity was sufficiently stable, 5 μ l CNO (at concentration of 1mM.) was injected at 0.2 μ l/min (25-min injection period) and the recording was stopped only 50min after starting CNO injection. We favored this approach because when the injection sites were too close of the recording sites and the injection flow rate higher (i.e., 1 μ l/min), CNO physically pushed away the recorded neurons leading to their lost. Importantly, to estimate the effective area of 5 μ l of CNO injected at 0.2 μ l/min, we injected (with the device and flow rate used in the current study) 5 μ l of Bromophenol blue 0.02 % in “phantom brain” models (with 0.6% agarose) that are presumed to replicate the properties of living brain tissue (Pomfret *et al.*, 2013). Moreover, to guarantee a complete CNO washout between the recordings, only one CNO injection was performed per day of recording.

Once the recording session completed, animals were kept under observation until they were fully awake. Note that no abnormal behavior was observed after full awakening. As controls, similar recordings following CNO injections were made in the contralateral/hM4Di DREADD-free GPe (i.e. GPe without viral vector injections - right and left GPe in monkeys F and E, respectively).

Data analysis

Single-unit pallidal activity was assessed by sorting spike trains from each 250-6000Hz band-pass filtered signal recorded within the GPe via offline spike detection and sorting method (Offline Sorter v4.4.2.0, Plexon Inc, Dallas, TX, USA). Threshold-crossing method for spike detection (negative voltage threshold trigger), principal component analysis for spike feature

extraction and K-means clustering method (K-Means Scan with number of clusters between 1 and 5) were applied.

The isolation quality of each sorted unit before, during and after CNO injection was systematically graded by calculating its isolation score (IS, Joshua *et al.*, 2007) for each 30s-bin. IS ranges from 0 (i.e. highly noisy) to 1 (i.e. perfect isolation). Quantification of the isolation quality of the sorted units is extremely important. Indeed, poor quality isolation of the sorted units affects the calculated discharge rate (Lewicki, 1998; Joshua *et al.*, 2007). False negative errors, namely missing real spikes, decrease the calculated discharge rate. On the other hand, false positive errors, namely erroneously classifying noise events as spikes, lead to artificial increase in the discharge rate of the detected unit.

Only sorted units with mean IS ≥ 0.6 (well-isolated units) over their full recording span (i.e., 120 of 30s-bins or 60min) were included in the database. Moreover, we also identified and excluded the 30s-bins where IS ≥ 2 or ≤ -2 SDs of the mean IS [i.e., $\sim 5\%$ (6/120) of the bin values were excluded per well-isolated unit, empirical 68-95-99.7 rule] in order not to erroneously bias the results with high or low firing rate due to the poor isolation quality of a unit during particular bins.

Then, we calculated the perievent time histogram (PETH) for each well-isolated unit. Since GPe neurons are GABAergic and possess local axon collaterals (Kita & Kitai, 1994; Bevan *et al.*, 1998; Sato *et al.*, 2000; Sadek *et al.*, 2007), it is likely that hM4Di DREADD-based inhibition of the GPe neurons located in the vicinity of the recorded neuron will reduce the lateral inhibition exert on the recorded neuron and will lead to some increases in its activity. A unit was therefore considered to globally modulate its activity during a specific period (i.e., during or after CNO injection) when its period-related activity (i.e., mean of all the bin values during the specific period) was ≥ 2 or ≤ -2 SDs of the baseline firing rate (i.e., mean of all bin values during the 10-min baseline period before CNO injection). Moreover, for each PETH, we also determined at each 30s-bin the polarity (increase vs. decrease) of the activity compared to its baseline and examined the period-related increases and decreases in activity. In doing so, a unit was considered to significantly increase or decrease its activity during a specific period when its period-related increase or decrease in activity (i.e., the mean of the bin values of the increases or decreases during the specific period) was ≥ 2 or ≤ -2 SDs of the baseline firing rate, respectively.

Histology and hM4Di DREADD extent analysis

At the end of the experimental sessions, animals were deeply anesthetized with a lethal dose of pentobarbital (50 mg/kg) and transcardially perfused with 0.9% physiological saline solution. The brain was removed for each monkey and the two hemispheres were divided and cut into three parts each. These tissues were post fixed in a large volume of 4% buffered

paraformaldehyde solution for one week at 4°C and cryoprotected in successive baths of 20% and 30% sucrose solution diluted in 0.1M phosphate-buffered saline (PBS) at 4°C until they sunk. Finally, brains were frozen by immersion in an isopentane bath at -55°C for 5 minutes and stored at -80°C. The entire BG (and more specifically the GPe) were cut in the coronal plane on a Leica 3050S cryostat into 50µm serial free-floating sections (24 series) collected in PBS-Azide 0.2% and store at 4°C until they were processed for mCherry immunoreactivity.

One series for each hemisphere was used for the staining. Briefly, endogenous peroxidases were first blocked by a peroxidase block reagent incubation of 10min (DAKO Peroxidase Block reagent, #S202386, Agilent, France). After 2 rinses with 0.1M PBS, sections were blocked in a solution of BSA 2%-TritonX100®0.3%-PBS 0.1M for 30 minutes before being incubated overnight in rabbit monoclonal anti-mCherry antibody (Abcam, ab213511; 1:1.000 dilution in BSA 0.2%-TritonX100®0.3%-PBS 0.1M). After rinsing sections with PBS, the staining was revealed by 30 min incubation in a HRP-anti rabbit polymer system (DAKO REAL Envision™ Kit, #K400311-2, Agilent, France) followed by a DAB revelation of few seconds (DAKO DAB Kit, #K346811-2, Agilent, France). Free-floating sections were mounted on gelatine-coated slides, counterstained with 0.2% cresyl-violet solution, dehydrated and coverslipped. Then, high-resolution whole slide color images were acquired with a Panoramic SCANNER (3D Histech, Hungary) at x20 magnification with 5 layers in extended picture. These high-resolution pictures were used to localize the injection and recording sites based on the Nissl stain which allowed the visualization of traces due to the passage of syringes and electrodes, and also to analyse hM4Di DREADD expression.

The extent of mCherry staining within the ipsilateral/hM4Di DREADD-transduced and contralateral/hM4Di DREADD-free GP (i.e., both the external and internal segments of the globus pallidus) was quantified with threshold surface analysis (Arotcarena *et al.*, 2019) using Mercator software (Mercator V7.13.4, Explora Nova, France). The mCherry fluorescent protein tag was fused to the hM4Di DREADDs; therefore the extent of mCherry staining reliably reflected the expression of hM4Di DREADDs. Briefly, GP was first delineated for each serial section of the series. The color thresholding tool of the software was used to select the threshold corresponding to the mCherry staining. The software extracted the surface corresponding to both the GP and the defined threshold, and the extent of mCherry staining was defined as the percentage of mCherry staining within the GP cross section surface. The same threshold surface analysis was performed for 2mm diameter circles centered on the presumed (visually identified on GP sections) viral vector injection sites in the ipsilateral/hM4Di DREADD-transduced GPe.

Software and Statistics

The data from the two monkeys were pooled since no contradictory results were detected between them. Nevertheless, the neuron characteristics of each monkey are given in table S1. All the data and statistical analyses were done using custom-made MATLAB R2016a routines (Mathworks, Natick, MA, USA). Wilcoxon signed rank tests and Mann-Whitney U-tests were used for statistical comparisons of paired and unpaired samples, respectively. Given the small sample sizes, Fisher's exact test was employed to compare proportions between samples. Statistics presented in this report, if not specified, use mean \pm standard error to the mean (SEM) and the criterion for statistical significance was set at $P < 0.05$ for all statistical tests.

Results

Database

We recorded 18 (11 and 7 in monkey F and E, respectively) and 8 (3 and 5 in monkey F and E, respectively) well-isolated GPe units ($IS \geq 0.6$) following local CNO injection in hM4Di DREADD-transduced and hM4Di DREADD-free GPe, respectively (table S1).

Fig.1D and E depicts two representative examples of neurons showing the evolution of their firing rates following CNO injection in hM4Di DREADD-transduced (Fig.1D) and hM4Di DREADD-free (Fig.1E) GPe. As illustrated, a substantial decrease in the firing rate was observed following local intra-GPe microinjection of CNO in the hM4Di DREADD-transduced GPe, while isolation quality of the unit remained stable over time (Fig.1D). In contrast, no consistent change in the firing rate was found following local intra-GPe microinjection of CNO in the hM4Di DREADD-free GPe, while isolation quality of the unit remained stable over time (Fig.1E). The firing rate of each GPe neuron recorded before, during and after CNO injection in the hM4Di DREADD-transduced and hM4Di DREADD-free GPe is illustrated in Fig.1F and G. As already described (DeLong, 1971; Elias *et al.*, 2007; Bugaysen *et al.*, 2010; Benhamou *et al.*, 2012) we found a spectrum of spontaneous (baseline) neuronal firing rates within the GPe (from low to high firing rate). However, although our samples were small, we did not find any obvious cell's firing rate-specific hM4Di DREADD's action/CNO's effect (Fig.1F and G).

Pallidal activity in the hM4Di DREADD-transduced and hM4Di DREADD-free GPe following CNO injections

Fig.2A and B (upper left panels) depicts the mean relative PETHs for hM4Di DREADD-transduced and hM4Di DREADD-free GPe, respectively. The mean relative PETH is calculated as the arithmetic average of all the Z-normalized PETHs and reflects the common assumption that the on-going activity of the studied population is close to zero and that the studied neurons excite their neuronal targets that act as integrate-and-fire neurons

(Izhikevich, 2007). For Z-score normalization (or standardization), we used the mean and SD firing rate of the 10-min baseline period before CNO injection. In doing so, we took into account the variability of the baseline firing rate in both the normalization process and the identification of the changes in activity following CNO injection.

In hM4Di DREADD-transduced GPe, 8/18 (44.4%) units modulated their period-related activity following CNO injection (3 during CNO injection, 4 after CNO injection and 1 during both periods, Fig.2A, upper middle panel), resulting in a significant reduction in the population activity after CNO injection compared to baseline ($-6.66 \pm 1.57\text{Hz}$, Wilcoxon signed rank test, $Z_{\text{val}} = 2.2428$, $P = 0.0249$, Fig.2A, upper right panel). In contrast, no unit modulated its period-related activity following CNO injection in hM4Di DREADD-free GPe (Fig.2B, upper middle panel). Consistently, we did not find any significant change in the population activity following CNO injection in hM4Di DREADD-free GPe (Fig.2B, upper right panel).

When examining time-varying increase/decrease balance of activity following CNO injection in the hM4Di DREADD-transduced GPe (Fig.2A, lower left panels), we found that 3/18 (16.7%) and 6/18 (33.3%) units exhibited a significant period-related increase and decrease in activity, respectively, during CNO injection (Fig.2A, lower middle panels). In addition, 8/18 units (44.4%, including the 6 units with significant decreased activity and 2 of the 3 units with significant increased activity during CNO injection) exhibited a significant period-related decrease in activity after CNO injection (Fig.2A, lower middle panel). These results suggest that neuronal activity of the well-isolated units exhibited diverse patterns in timing and polarity (increase/decrease) of firing rate modulations during and after CNO injection. Nevertheless, significant decreases in activity were more frequent and also more pronounced than significant increases in activity ($-13.06 \pm 2.71\text{Hz}$ vs. $+10.27 \pm 4.19\text{Hz}$ during CNO injection and even became exclusive after CNO Injection ($-13.42 \pm 4.75\text{Hz}$, Fig.2A, lower right panel). In contrast, only one unit exhibited a significant period-related increase in activity after CNO injection in hM4Di DREADD-free GPe ($+9.44\text{Hz}$, Fig.2B, lower left, middle and right panels). Overall, the number of units exhibiting a significant period-related decrease in activity following CNO injection were significantly larger in hM4Di DREADD-transduced GPe than in the hM4Di DREADD-free GPe [8/18 (44.4%) vs. 0/8 (0%) Fisher's exact test, $p = 0.031$].

DREADD expression and recording-CNO injection sites within GP

Extent of mCherry staining (based on the threshold surface analysis, see *Histology and hM4Di DREADD extent analysis* in Materials and Methods) within the ipsilateral/hM4Di DREADD GP of monkey F is represented in Fig.3A. Threshold surface analysis revealed that mCherry staining surface in the ipsilateral/hM4Di DREADD GP was larger around AC0, AC-1 and AC-2 (and maximal at AC-1, Fig.3B). Also, when examining mCherry staining in the

vicinity of the viral vector injection sites located in the GPe (2mm diameter circles centered on the viral vector injection sites) mCherry staining surface reached up to ~24% (Fig.3C, left). Moreover, we observed a substantial number of mCherry-labeled GPe neurons (Fig.3C, right). Although the viral titer was lower (3×10^{12} viral particles/ml) in monkey F (one order of magnitude less than the viral titer used in monkey E), these results therefore indicate that hM4Di DREADDs fused to mCherry were expressed at high level in the GPe neurons located in the vicinity of the viral vector injection sites. On the contrary, the threshold surface analysis demonstrated that mCherry staining surface and hM4Di DREADD expression in the contralateral GPe was weak (close to 0%) compared to the results in the ipsilateral/hM4Di DREADD-transduced GPe (Fig.S1), thus confirming that the contralateral GPe was hM4Di DREADD-free.

Finally, examination of the 3D spatial layout of the recording and CNO injection sites in the ipsilateral/hM4Di DREADD-transduced and contralateral/hM4Di DREADD-free GPe revealed that both the recording and CNO injection sites were located between AP 18 (AC0) and 16 (AC-2), between L9 and L5, and between D17 and D15 (Fig.3D and E) which corresponds to the area of the ipsilateral/hM4Di DREADD-transduced GPe with the highest density of hM4Di DREADDs (Fig.3A, B and C). Moreover, it shows that the distance between the recording and CNO injection sites were not significantly different between the ipsilateral/hM4Di DREADD-transduced and contralateral/hM4Di DREADD-free GPe (1.32 ± 0.07 mm and 1.39 ± 0.08 mm, respectively, Mann-Whitney U-tests, $Z_{val} = 0.56$, $P = 0.58$, Fig.3D and E).

Discussion

This study is the first to demonstrate *in vivo* DREADD-based modulation of the neuronal activity in the NHP. Our results reinforce the view that DREADD technique can be used as a powerful tool to remotely, reversibly and repeatedly manipulate the neural mechanisms underlying higher brain functions, not only in rodents, but also in NHPs. Besides, given the unique value of NHP models in translational research, our results are more easily translatable to human. In neurosciences, traditional and available techniques in NHPs to examine the causal link between structure and function are based on lesions or pharmacological approaches which are highly invasive, if not irreversible. Validation of the DREADD technique in NHP model is therefore fundamental for basic and translational neuroscience research.

Potential limitations

In the current study, the spatial arrangement of the recording-CNO injection sites within the GPe (Fig.3D and E) made the interpretation of the results complicated. Indeed, it is difficult to precisely estimate the effective area of 5 μ l of CNO injected at 0.2 μ l/min in living brain tissue

and CNO might not diffuse far enough to impact the activity of the recorded neurons. Nevertheless, dye injection in “phantom brain” models (see *Electrophysiology and intracerebral injections* in Materials and Methods for details) suggested that the injection of 5 μ l of CNO at 0.2 μ l/min produced a sphere centered on the tip of the needle with ~2mm radius after 25min (i.e., when injection stops) and ~2.5mm radius after 50min (i.e., when recording stops). Therefore, the GPe neurons (recorded within > 2mm radius from the tip of the needle, Fig.3D and E) were most certainly exposed to CNO after 25min of CNO injection at 0.2 μ l/min (i.e., when CNO injection stops). We found that the average distance between the recording and CNO injection sites was 1.32 ± 0.07 mm and 1.39 ± 0.08 mm in the ipsilateral/hM4Di DREADD-transduced and contralateral/hM4Di DREADD-free GPe, respectively (Fig.3D and E). Moreover, although GPe has an irregular shape in each dimension, the volume of the NHP GPe [~ 250 mm³ (Hardman *et al.*, 2002)] is much larger than a sphere of ~2mm or ~2.5mm radius (~ 35 mm³ or ~ 65 mm³, respectively). Therefore, we can exclude the possibility that the modulations in pallidal activity following CNO injection were due to the spread of the CNO to neighboring regions or to multi-synaptic network effects. Equally important, GPe neurons possess local collateral axons (Kita & Kitai, 1994; Bevan *et al.*, 1998; Sato *et al.*, 2000; Sadek *et al.*, 2007). It is estimated that each local collateral axon forms more than 650 synaptic connections within the GPe (Hegeman *et al.*, 2016). Moreover, it appears that connections are ideally located on somas and proximal dendrites to strongly affect the firing of their postsynaptic targets (Bugaysen *et al.*, 2013). Given the GABAergic nature of the GPe neurons, their local collaterals probably exert a powerful lateral inhibition within the GPe. Therefore, early inhibition of hM4Di DREADDs-transduced GPe neurons closer to the CNO injection sites than the recorded neurons (i.e., neurons in the immediate vicinity of the CNO injection sites) could produce a reduction of the lateral inhibition and lead to early increases in the activity of the recorded neurons. Moreover, it is likely that decreases in activity following CNO injections were also lessened due to a reduction of the lateral inhibition in the vicinity of the recorded neurons. In line with this view, we observed that neurons in hM4Di DREADD-transduced GPe exhibited diverse timing and directional (increase or decrease) patterns of discharge modulations following local CNO microinjection. Nevertheless, we demonstrated that decreases in activity prevailed in the hM4Di DREADD-transduced GPe during CNO injection and became even exclusive after CNO injection (Fig.2A).

Another possible limitation is that we did not inject a viral vector not expressing the hM4Di DREADDs (e.g. AAV-hSyn-mCherry) into the contralateral GPe. Therefore, we cannot rule out the possibility that AAV transduction itself (i.e., transduction of the neurons with AAV not expressing the hM4Di DREADDs) could mediate modulations in activity of the transduced GPe neurons in response to CNO injection. Nevertheless, it is unlikely because the CNO's

action is receptor binding-dependent (Roth, 2016). Moreover, even though AAV transduction itself could modify the spontaneous activity of the transduced GPe neurons, the responses to CNO were defined as the deviation to the baseline firing rate (10min period before CNO injection) and therefore the possible effect of the AAV transduction itself on the spontaneous activity of the transduced GPe neurons was not informative.

Chemogenetic (hM4Di DREADD-based) vs. pharmacological inhibition of the NHP GPe neurons

Decreases in NHP pallidal activity has already been reported following local microinjections of muscimol [i.e., ionotropic GABA_A receptor agonist (Baron *et al.*, 2002; Galvan *et al.*, 2005)] or baclofen [i.e., a metabotropic GABA_B receptor agonist (Galvan *et al.*, 2005)]. Moreover, local pharmacological modulations of the AMPA/kainate or NMDA receptors also provoke decreases in the activity of the GPe neurons (Kita *et al.*, 2004). Classic pharmacological modulations of the GABAergic and glutamatergic transmission can therefore strongly reduce the NHP pallidal activity (between ~-30% and ~-60% decrease). Given the different drug injection procedures (e.g., types and concentrations of drugs, volumes and rates of injection, and spatial arrangements of the recording-CNO injection sites) and the different data analysis methods (e.g., detection/calculation of the significant decreases in activity and assessment of the isolation quality of the units over time), the comparison of the drug's effect on the pallidal activity between studies is difficult - especially since drug's effect in our study (CNO's effect) also depended on hM4Di DREADD expression. Moreover, we recorded the pallidal activity in anesthetized animals. Earlier studies demonstrated that anesthesia reduces the discharge rate of the GPe neurons [e.g.,(Castrioto *et al.*, 2016)]. Therefore, we cannot rule out the possibility that anesthesia regimen minimized the DREADD's action/CNOs 'effect on pallidal activity. Nevertheless, the mean firing rate during the significant decreases observed in hM4Di DREADD-transduced GPe during and after CNO injection (6/18 and 8/18 units, respectively) still reduced by on average ~13Hz compare to the baseline (Fig.2A, lower right panel) - that is ~-30% and ~-45% decrease during and after CNO injection, respectively (table S1).

No clear hM4Di DREADD-independent modulations in pallidal activity following local CNO injection in NHP GPe

When CNO is systemically injected, its use is problematic. Indeed, CNO hardly crosses the blood-brain barrier (Gomez *et al.*, 2017; Raper *et al.*, 2017) and can be *in vivo* converted to clozapine (Jann *et al.*, 1994; Chang *et al.*, 1998; MacLaren *et al.*, 2016; Gomez *et al.*, 2017; Raper *et al.*, 2017) which shows high blood-brain permeability and high affinity to muscarinic-

based DREADDs (Ji *et al.*, 2016; Gomez *et al.*, 2017). However, clozapine is capable of binding to a variety of endogenous receptors (Galvan *et al.*, 2018).

Here, our goal was to unambiguously show (using a straightforward strategy) that hM4Di DREADDs can reduce firing rate of NHP GPe neurons when activated by a specific ligand. This is why we favored local intracerebral CNO microinjections to make sure our results were not confounded by endogenous non-DREADD target actions of the clozapine. However, since high CNO concentration (1mM) can activate endogenous receptors (Gomez *et al.*, 2017) and induce DREADD-independent responses following local CNO injections, we also performed neuronal recordings following local CNO injections in the contralateral/hM4Di DREADD-free GPe (i.e. GPe without viral vector injections). Immunohistochemistry results in the contralateral/hM4Di DREADD-free GPe indicated that hM4D-DREADD expression in contralateral GPe was weak (close to 0%,) compared to the ipsilateral/hM4Di DREADD-transduced GPe (Fig.S1) in which hM4Di DREADDs were expressed at high level in the GPe neurons located in the vicinity of the viral vector injection and recording sites (Fig.3A, B and C). In doing so, we found no robust modulations of activity in the contralateral/hM4Di DREADD-free GPe following CNO injection (Fig.2B), thus revealing no off-target effects of local injections of CNO (even at 1mM) on the NHP pallidal activity and validating hM4Di DREADD-based inhibition of NHP pallidal neurons. These results are in line with several studies in rodents that reported behavioral effectiveness (with no DREADD-independent behavioral effects) of *in vivo* 1mM CNO microinjection in ventral tegmental area (Mahler *et al.*, 2014), lateral septum (McGlinchey & Aston-Jones, 2018), dorsal hippocampus (Ge *et al.*, 2017), or orbital cortex (Lichtenberg *et al.*, 2017). Therefore, it supports the idea that the presence of receptors susceptible to nonspecific CNO/clozapine binding may be brain region-dependent (Mahler & Aston-Jones, 2018).

Significance and perspectives

Our post-mortem histochemical did not allow us to examine the subcellular localization of the DREADDs (i.e., intracellular space vs. plasma membrane). In a recent NHP study, Galvan and colleagues demonstrated that the pattern of subcellular expression of DREADDs in monkeys depends on the DREADD/tag combination (Galvan *et al.*, 2019). In particular, they showed that using hM4Di DREADD fused to mCherry (as we did here) leads to a low proportion of hM4Di at the plasma membrane of the monkey, but not the rodent, neurons. In contrast, using the same hM4Di DREADD fused to the HA-tag leads to a robust plasma membrane expression in both monkey and rodent neurons. However, since behavioral effect has already been observed in monkeys using the hM4Di-mCherry combination (Grayson *et al.*, 2016; Upright *et al.*, 2018), it is possible that a small fraction of hM4Di at the plasma membrane is sufficient for decreasing the activity of hM4Di DREADD-transduced neurons.

In the current study, we used high concentration of CNO (1mM) that can activate endogenous receptors (Gomez *et al.*, 2017). Unfortunately, we did not test lower concentrations of CNO (<1mM), nor did we try new DREADD ligands (i.e., JHU37152, JHU37160 or Deschloroclozapine) which show excellent brain penetrance and high *in vivo* DREADD potency - including in NHP (Bonaventura *et al.*, 2019; Nagai *et al.*, 2019). Moreover, we recorded a relatively small number of GPe neurons (especially in the control condition) including some that exhibited fluctuations in their baseline activity. Therefore, our results should be interpreted with cautious and further NHP studies must be conducted on larger samples of neurons using different DREADD/tag combinations after local and systemic injections of the new effective DREADD ligands (Bonaventura *et al.*, 2019; Nagai *et al.*, 2019) to conclude on the capacity of the DREADDs for modulating NHP neuronal activity. Nevertheless, this study is still the first to explore the effect of DREADD ligands on the (pallidal) neuronal activity of the NHP and represents a step closer the validation of DREADD technology in NHPs.

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Competing Interests

The authors declare no conflict of interest.

Data Accessibility

The data are available from the corresponding author upon request.

Author Contributions

M.D., E.B., T.B. designed the research; M.D., T.H.N., H.O., N.B., S.D. performed the research; M.D., analyzed the data; M.D., T.B. wrote the paper; All authors read and approved the final version.

Abbreviations

AAV, adeno-associated virus; CNO, clozapine-N-oxide; DREADDs, Designer Receptors Exclusively Activated by Designer Drugs; GPe, external globus pallidus; IM, intramuscular;

IS, isolation score; NHP, non-human primate; PETH, perievent histogram; PDS, phosphate-buffered saline; SD, standard deviation; SEM, standard error to the mean

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Figure legends

Fig.1. Recording configuration and examples. (A) Coronal and (B) sagittal X-ray images of the animal in the stereotaxic frame showing the apparatus during GPe unit recordings. (C) Representative coronal section - 1 mm from the anterior commissure (adapted from Martin & Bowden, 2000) indicating the spatial arrangement of the electrode and needle tips during neuronal recordings in the GPe. (D and E) Peri-event time histograms (PETHs) of two representative GPe units recorded following local CNO microinjection in hM4Di DREADD-transduced (D) and hM4Di DREADD-free (E) GPe. Black and red traces indicate the evolution of the discharge rate (left y-axis) and isolation score (IS, right y-axis) of the unit before, during and after CNO injection. 0 and 25s correspond to CNO injection start and end (5 μ l of 1mM CNO at 0.2 μ l/min). (F and G) Discharge rate of the individual neurons before, during and after CNO injection in the hM4Di DREADD-transduced and hM4Di DREADD-free GPe. N indicates the number of units recorded in the hM4Di DREADD-transduced and hM4Di DREADD-free GPe.

Fig.2. Injection data analysis. Effect of a local CNO microinjection on the pallidal activity in (A) hM4Di DREADD-transduced and (B) hM4Di DREADD-free GPe. **Left panels**, Z-score normalized population change in activity (**upper panels**) and Z-score normalized population increases and decreases in activity (**lower panels**) in response to CNO injection. 0 and 25s correspond to CNO injection start and end (5 μ l of 1mM CNO at 0.2 μ l/min). **Middle panels**, Box plots showing the Z-score normalized change in activity (**upper panels**) and Z-score normalized increases or decreases in activity (**lower panels**) of each unit during and after CNO injection. In each box, the bold line indicates the median, and the bottom and top edges of the box indicate the 25th and 75th percentiles, respectively. **Right panels**, Bar plots showing the mean changes in activity (in Hz) of all the units (**upper panels**) and mean increases and decreases in activity (in Hz) of the units exhibiting a significant period-related increase and decrease (**lower panels**) during and after CNO injection. For each unit, change in activity corresponds to the period-related activity (i.e., mean of all the bin values during a specific period) after subtracting the firing rate during the 10-min baseline period before CNO injection (i.e., mean of all bin values during the 10-min baseline period before CNO injection). Increase and decrease in activity correspond to the period-related increase and decrease in activity, respectively, (i.e., the mean of the bin values of the increases or decreases during the specific period) after subtracting baseline firing rate. N indicates the number of units

significantly increasing or decreasing their activity during the different periods. Error bars represent SEMs.

Fig.3. hM4Di DREADD expression and recording-CNO injection site reconstructions within the GPe. (A) Antero-posterior representation of the extent of mCherry staining within the ipsilateral/hM4Di DREADD GP of monkey F (using threshold surface analysis, scale bar: 2000 μ m). **(B)** Illustration of mCherry staining patterns at AC0, AC-1 and AC-2 (left, scale bar: 2000 μ m) and quantification of mCherry staining surface for the different GP sections (right). Asterisks indicate the three sections with the highest mCherry staining surface in GP, namely AC0, AC-1 and AC-2. **(C)** Microscopic illustrations (yellow and pink boxes) of the density of mCherry-labeled GPe neurons located in the vicinity of two viral vector injection sites [2mm diameter circles (black) centered on the viral vector injection sites]. Scale bar: 2000 μ m (left) and 50 μ m (right). **(D and E)** 3D spatial layout of the recording and CNO injection sites in the ipsilateral/hM4Di DREADD-transduced and contralateral/hM4Di DREADD-free GPe. Each black line links the recording site (red square) with its corresponding CNO injection site (blue circle). The coordinates of the recording and injection sites are given (in mm) from the vertical plane passing through the interaural line (AP), the midline (L) and the horizontal plane passing through the interaural line (D). Abbreviations: AC, anterior commissure; Cd, caudate nucleus; IC, internal capsule; GP, globus pallidus; GPe-i, external and internal globus pallidus; Pu, putamen.