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A Comparative Study of the Pharmacokinetics of Clozapine *N*-Oxide and Clozapine *N*-Oxide Hydrochloride Salt in Rhesus Macaques

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ABSTRACT

Translating chemogenetic techniques from nonhuman primates to potential clinical applications has been complicated in part due to in vivo conversion of the chemogenetic actuator, clozapine *N*-oxide (CNO), to its pharmacologically active parent compound, clozapine, a ligand with known side effects, including five boxed warnings from the Food and Drug Administration. Additionally, the limited solubility of CNO requires high concentrations of potentially toxic detergents such as dimethylsulfoxide (DMSO). To address these concerns, pharmacokinetic profiling of commercially available CNO in DMSO (CNO-DMSO, 10% v/v DMSO in saline) and a water-soluble salt preparation (CNO-HCl, saline) was conducted in rhesus macaques. A time course of blood plasma and cerebrospinal fluid (CSF) concentrations of CNO and clozapine was conducted (30–240 minutes post-administration) following a range of doses (3–10 mg/kg, i.m. and/or i.v.) of CNO-DMSO or CNO-HCl. CNO-HCl resulted in 6- to 7-fold higher plasma concentrations of CNO compared to CNO-DMSO, and relatively less clozapine (3%–5% clozapine/CNO in the CNO-DMSO group and 0.5%–1.5% clozapine/CNO in the CNO-HCl group). Both groups had large between-subjects variability, pointing to the necessity of performing individual CNO pharmacokinetic studies prior to further experimentation. The ratio of CNO measured in the CSF was between 2% and 6% of that measured in the plasma and did not differ across drug preparation, indicating that CSF concentrations may be approximated from plasma samples. In conclusion, CNO-HCl demonstrated improved bioavailability compared with CNO-DMSO with less conversion to clozapine. Further investigation is needed to determine if brain concentrations of clozapine following CNO-HCl administration are pharmacologically active at off-target monoaminergic receptor systems in the primate brain.

Introduction

In neuroscience, the most widely used chemogenetic manipulations are comprised of Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) (Farrell and Roth, 2013; Sternson and Roth, 2014; Urban and Roth, 2015). DREADDs are mutated versions of naturally occurring G-protein-coupled receptors that have lost their affinity for their native ligand and instead are activated by a designer actuator (Armbruster et al., 2007; Lee et al., 2014; Urban and Roth, 2015; Roth, 2016). One important feature of all DREADDs in studies of brain function is that the designer actuators are peripherally bioavailable, allowing for reversible manipulation of specific cell populations without the need to maintain indwelling cannula (Armbruster et al., 2007; Urban and Roth, 2015; Roth 2016). While this technology has a growing literature in rodent models, it has been used in only a small number of nonhuman primate studies, all published within the last 3 years (Eldridge et al., 2016; Grayson et al., 2016; Nagai et al., 2016; Galvan et al., 2018). Transfer of DREADD technology to the nonhuman primate brain is desirable because it would allow for cell type–specific targeting in an animal model with high anatomic and functional similarity to the human brain. One additional advantage of nonhuman primates is their longevity, allowing

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ABBREVIATIONS: AUC, area under the curve; CL, clearance; CSF, cerebrospinal fluid; CNO, clozapine *N*-oxide; DMSO, dimethylsulfoxide; DREADDs, Designer Receptors Exclusively Activated by Designer Drugs; 5-HT, 5-hydroxytryptamine; V_d, volume of distribution.

for longitudinal studies of complex behavior. Therefore, DREADDs have the potential to characterize circuit mechanisms mediating complex behaviors and maximize the potential of the nonhuman primate to inform translational applications in neurologic or psychiatric medicine.

When comparing DREADD techniques between rodents and nonhuman primates, a main concern has been the administration of the actuator, clozapine *N*-oxide (CNO). CNO is the primary ligand for the widely used DREADD receptors (M1, M2, M3, M4, and M5 mutant muscarinic receptors; chimeric M3 from adrenergic receptor; M3DqR165L; hM4D-neurexic variant). CNO is one of the major metabolites of the atypical antipsychotic clozapine through cytochrome P450 systems, primarily at the CYP1A2 isoform in humans (Doude van Troostwijk et al., 2003). Shortly following CNO administration, there can be measurable concentrations of clozapine in the blood and cerebrospinal fluid (CSF) due to rapid reverse metabolism from CNO to clozapine, a phenomenon originally reported in guinea pigs and humans (Jann et al., 1994), and later reported in macaques and rodents (MacLaren et al., 2016; Gomez et al., 2017; Raper et al., 2017; Manvich et al., 2018). Since clozapine is pharmacologically active at several monoaminergic receptor systems, including dopaminergic, serotoninergic, and adrenergic receptors (Bymaster et al., 1996; Selent et al., 2008), it is important to quantify the concentration of clozapine in the plasma and CSF following CNO administration.

CNO is not readily water soluble, and thus is frequently suspended in dimethylsulfoxide (DMSO) as a vehicle for peripheral administration. Because DMSO can be an irritant, the injection volumes must be large to accommodate a safe concentration of DMSO [i.e., the 10 mg/kg dose for 10 kg monkeys is 10 ml, as used in Eldridge et al. (2016) and Raper et al. (2017)]. To circumvent this concern, a salt form of CNO (CNO-HCl) was developed and tested in this study, capitalizing on the ability to conduct repeated measures of blood and CSF in the macaque monkey. A previous CNO pharmacokinetics study in rhesus macaques only tested CNO-DMSO and concluded that minimal concentrations of CNO are detectible in the CSF, calling into question the mechanism of DREADD receptor activation in macaques (Raper et al., 2017). The present study compares the bioavailability of CNO-DMSO to CNO-HCl in a dose- and time-dependent manner in plasma and CSF to examine brain penetrance and the ratio of clozapine:CNO in both body compartments.

Materials and Methods

Animals

Twelve rhesus macaques (*Macaca mullata*; 1 female and 11 males) aged 4–10 years old were used in this study (Table 1). All monkeys were born and raised at the Oregon National Primate Research Center (Beaverton, OR). All monkeys were under constant temperature (20–22°C) and humidity (65%) with free access to food and water on a 12/12-hour light/dark cycle (lights on at 7 AM). Monkeys were weighed weekly without sedation and monitored throughout the experiment by veterinary staff. All procedures were conducted in accordance with the National Institutes of Health (Bethesda, MD) and the Guide for the Care and Use of Laboratory Animals (https://grants.nih.gov/grants/olaw/guide-for-the-care-and-use-of-laboratory-animals.pdf), and were approved by the Oregon National Primate Research Center Institutional Animal Care and Use Committee.

CNO Preparation

All drugs were prepared fresh on the morning of each experiment. CNO and CNO-HCl were stored at room temperature and protected from light and moisture.

CNO-DMSO. CNO (MW: 342.82) was obtained from National Institutes of Health Rapid Access to Interventional Development (Bethesda, MD) and Toronto Research Chemicals (North York, Ontario, Canada). Prior to injection, CNO was initially suspended in a minimal volume of DMSO (Sigma-Aldrich, St. Louis, MO) at concentrations up to 100 mg/ml. Saline (0.9%) was added to the CNO and DMSO solution to achieve a final 10% (v/v) DMSO in saline solution (CNO concentration: 6.5 mg/ml). The drug was then passed through a 20 μm filter (Millipore, Burlington, MA) into a sterile vial before being pulled into individual sterile syringes for administration. This drug preparation of CNO will be referred to as "CNO-DMSO."

CNO-HCl. The same stock of CNO described previously was converted to CNO-HCl (379.29 mol. wt.). The CNO-HCl was dissolved in sterile saline (0.9%) to achieve a CNO concentration between 30 and 46 mg/ml; due to the inconsistency in solubility of the initial dosing solution of 46 mg/ml, it was decreased to 30 mg/ml for the remaining doses (Table 1). CNO-HCl was then passed through a 20 μm filter (Millipore) into a sterile vial before being drawn into sterile syringes for administration. This drug preparation of CNO will be referred to as "CNO-HCl."

CNO Dosing

Prior to all pharmacokinetics studies, monkeys were fasted overnight, beginning at 4 PM on the day prior to dosing. Water was available ad libitum throughout the experiment. CNO-DMSO and CNO-HCl were each administered both intramuscularly and intravenously on separate testing days (Table 1). Different tests of CNO

TABLE 1
Experimental time points

Drug Preparation/Dose	Concentration	Route	Blood Collection	CSF Collection
mg/kg	*mg/ml*		*min*	*min*
CNO-DMSO				
3.0	6.5	Intramuscular	60–240	120
5.0	6.5	Intramuscular	60–240	120
7.0	6.5	Intramuscular	60–240	120
6.5	6.5	Intravenous	45–60	45–60
CNO-HCl				
3.0	30	Intramuscular	30–240	—
5.6	46[a]	Intramuscular	30–240	—
10.0	30	Intramuscular	30–240	—
5.6	30	Intravenous	30	30

[a]Five out of six subjects received 5.6 mg/kg at the higher concentration of 46 mg/ml before the remaining doses were lowered to 30 mg/ml.

within the same monkey were at least 1 week apart. For all intramuscular doses, injections were maintained below 2 ml per injection site according to the Oregon National Primate Research Center institutional guidelines. For CNO-DMSO, injection volumes were up to 8 ml over four injection sites (bilateral quadriceps and biceps). For CNO-HCl, injection volumes were up to 3 ml over two injection sites (bilateral quadriceps). For intravenous injections, monkeys were first sedated with ketamine (10 mg/kg, i.m.) and then maintained on isofluorane (1% to 2%). Injections were made through a catheter placed in the saphenous vein and anesthesia was maintained until blood and CSF collection was complete.

Blood and CSF Collection

Following CNO injection, blood samples (1 to 2 ml in K2-EDTA tubes; BD, Franklin Lakes, NJ) were collected from the femoral vein while animals were under mild restraint in a bleeding tower or primate chair. A summary of experimental time points can be found in Table 1.

Blood samples were collected at 30, 60, 90, 120, 150, 180, and/or 240 minutes after injection. These time points were determined from a preliminary study conducted in the CNO-DMSO group, which included samples between 1 and 24 hours following drug administration. In this experiment, plasma CNO had dropped to near zero in all doses tested by the 5-hour time point (data not shown). In some instances, ketamine (2.9–10 mg/kg, i.m.) was used to sedate for safe blood collection. Previous studies have indicated that there is no significant effect of sedation on CNO pharmacokinetics in macaques (Raper et al., 2017).

For CSF collection, monkeys were sedated with a combination of telazol (tiletamine/zolazepam; 2.5–4 mg/kg, i.m.), carprofen (4 mg/kg, s.c.), and/or flumazenil (3.4–11.6 μg/kg, i.v.) or isofluorane (1% to 2%) and positioned in lateral recumbency with head held in flexion. A sterile prep of the posterior neck was performed, and a 0.5–1.0 ml sample of CSF was obtained via percutaneous cisternae access with a 23-gauge needle and collected in a 15 ml conical tube (Fisher Scientific, Pittsburgh, PA). When subsequent blood samples were collected, monkeys were recovered for later time points (Table 1).

Blood samples were kept on ice and then centrifuged at 1811*g* at 4°C for 15–20 minutes (581OR; Eppendorf, Happauge, NY). Plasma was transferred to screw top freezer tubes (Sarstedt, Rommelsdorfer Strasse, Germany) and stored at −80°C until ready for assay. CSF was transferred directly to storage at −80°C until ready for assay. If contaminating blood was present in the CSF sample, it was centrifuged at 201*g* at 4°C for 5 minutes to pellet red blood cells. The supernatant was then collected and transferred to screw top freezer tubes and stored at −80°C for further analysis.

Preparation of Samples and Calibrators for CNO and Clozapine Measurement

The analysis of clozapine and clozapine *N*-oxide was adapted from the method described by Wohlfarth et al. (2011). Concentrated stocks of clozapine-d8 (Toronto Research Chemicals) and clozapine *N*-oxide (Tocris, Minneapolis, MN) were prepared at 10 mg/ml in DMSO and the internal standard, clozapine-d_8, was prepared at 1 mg/ml in DMSO. Working dilutions were made in methanol (Burdick and Jackson, Muskegon, MI) and stored at −80°C. The clozapine-d_8 working solution was prepared in methanol at 100 ng/ml. In order to bracket the entire range of analyte concentrations, two separate calibrator sample sets were prepared in CSF and plasma, each at five different concentrations. The low concentration was from 0.05 to 25 ng/ml and the high calibration standards ranged from 25 to 5000 ng/ml. Plasma, CSF, and calibrator samples were processed as follows: 500 μl of plasma or CSF was transferred to a glass tube followed by the addition of 5 μl of the internal standard stock (100 ng/ml), 1 ml of sodium carbonate (Fluka, Buchs, Switzerland) solution (100 g/l water), and finally 3 ml of ethyl acetate. Samples were vortexed, centrifuged at 2000*g* to separate the phases, and the ethyl acetate was transferred to a glass tube. The solvent was evaporated to dryness using a speed vacuum concentrator at room temperature. The dried samples were reconstituted in 100 μl of methanol, mixed, and then filtered using a 0.22 μm centrifugal filter and transferred to autosampler vials for liquid chromatography–tandem mass spectrometry analysis. For the high concentration samples and calibrators, the samples were reconstituted in methanol and then diluted 1:10 prior to analysis.

Extracts were analyzed using a 4000 QTRAP hybrid/triple quadrupole linear ion trap mass spectrometer (AB SCIEX, Framingham, MA) with electrospray ionization in positive mode. The mass spectrometer was interfaced to a Shimadzu (Columbia, MD) SIL-20AC XR autosampler followed by two Shimadzu LC-20AD XR LC pumps. The instrument was operated with the following source settings: source voltage 5000 V, ion source gas 1 (GS1) 50, ion source gas 2 (GS2) 50, curtain gas (CUR) 10, temperature (TEM) 600, and collision gas (CAD) medium. The multiple reactions monitoring transitions monitored with a 150-millisecond dwell time were optimized by direct infusion of the compounds individually and are listed in Table 2, where the transition used for quantification is shown in bold. A gradient mobile phase was delivered at a flow rate of 0.4 ml/min and consisted of two solvents, 1 mM ammonium formate (Sigma-Aldrich) in water (A), and methanol (B). The initial concentration of B was 20%, followed by an increase to 95% B over 5 minutes, held at 95% for 2 minutes, and decreased to 20% again in 0.1 minute, followed by re-equilibration for 2.9 minutes. Flow was diverted away from the source except for the period from 2.5 to 5 minutes. The column used was a Synergi 4μ Max-RP 80A 150 × 2 mm column (Phenomenex, Torrance, CA) with a BetaBasic C18 guard column (2 × 10 mm; ThermoFisher, Waltham, MA) maintained at 35°C using a Shimadzu CTO-20AC column oven.

Instrument control and data were acquired and analyzed using Analyst 1.6.2 software (SCIEX, Framingham, MA). The lower limit of quantification for clozapine and clozapine *N*-oxide from plasma and CSF was 0.05 ng/ml. The accuracy was 106% or greater and the precision was less than 5% for both compounds with signal-to-noise ratios of greater than 10. The slopes of standard curves for each analyte were the same when prepared from CSF or plasma.

Data Analysis

For each blood and CSF sample, a percentage of the amount of clozapine to CNO was calculated [within-sample percentage of clozapine/CNO = (plasma clozapine, ng/ml)/(plasma CNO, ng/ml)].

For plasma CNO concentration results, the common time points collected between the CNO-DMSO and CNO-HCl groups (60-, 90-, and 240-minute samples) were used for further pharmacokinetic analysis using the NonCompart package in R (https://cran.r-project.org/web/packages/NonCompart/NonCompart.pdf). For each subject and dose,

TABLE 2

Compound-specific multiple reactions monitoring transitions for the analysis of CNO and clozapine optimized for direct infusion of authentic standards

The Q1 mass was the parent mass and the Q3 mass was the product mass that was monitored and quantified. The entries presented in bold indicate the transition was used for quantitation, while the nonbold entries indicate the qualifier transition. The declustering potential, entrance potential, collision energy, and collision cell exit potential were all tuned for each compound and measured in voltage.

Q1 Mass	Q3 Mass	Compound	DP	EP	CE	CXP
327.1	**270**	**Clozapine**	**96**	**10**	**33**	**6**
327.1	192	Clozapine	96	10	63	12
335.1	**275**	**Clozapine-d_8**	**96**	**10**	**35**	**20**
335.1	192.1	Clozapine-d_8	96	10	63	12
343.1	**192**	Clozapine N-oxide	**81**	**10**	**65**	**12**
343.1	256	Clozapine N-oxide	81	10	29	18

DP, declustering potential; EP, entrance potential; CE, collision energy; CXP, collision cell exit potential.

the following parameters were calculated: area under the curve (AUC), peak plasma concentration (C_{max}), time of peak plasma concentration, terminal half-life, observed volume of distribution (V_d), and observed clearance (CL). The AUC was calculated between 60 and 240 minutes (AUC_t) using the trapezoid rule and served as the primary dependent measure.

One-way repeated measures analyses of variance were conducted separately for CNO-DMSO and CNO-HCl groups to determine the effect of dose on plasma concentrations of CNO and clozapine as well as the percentage of clozapine to CNO in plasma (percentage of clozapine/CNO). Next, plasma concentrations of CNO and clozapine as well as the percentage of clozapine/CNO were compared between CNO-DMSO and CNO-HCl using the results from 3.0 mg/kg. To account for differences in sample size, unpaired Welch's t tests were used for across-group analyses (CNO-DMSO: $n = 2$; CNO-HCl: $n = 6$). CNO, clozapine, and percentage of clozapine/CNO were directly compared between intramuscular and intravenous routes of administration for the CNO-HCl group ($n = 6$ to 7) using a mixed-effects model. Additional comparisons were made between the relative amount of plasma CNO compared to CSF CNO between the CNO-DMSO and CNO-HCl groups (one-way analysis of variance).

Results

Plasma Pharmacokinetics of CNO-DMSO and CNO-HCl (Time Course and Dose Effects). CNO-DMSO and CNO-HCl both resulted in increases in plasma levels of both CNO and clozapine when administered intramuscularly (Fig. 1, A–D). In general, CNO reached peak concentrations in plasma between 30 and 90 minutes, and then decreased as a function of time, with CNO still at detectable concentrations 4 hours following drug administration (Fig. 1A). Clozapine levels, on the other hand, rose slowly over the 4-hour period with peak concentrations either at 90 or 240 minutes (Fig. 1B). Plasma concentrations from the 60-, 90-, and 240-minute time points following drug administration were used for dose comparisons and to compare across the two forms of the drug. Mean peak concentrations (C_{max}) of CNO in the plasma following CNO-DMSO after 3.0, 5.0, and 7.0 mg/kg were 226, 415, and 595 ng/ml, respectively, but the variance also increased greatly with dose (Table 3). A repeated measures analysis of variance of plasma CNO AUC by dose was not significant, likely due to high variability ($P = 0.24$). In the CNO-HCl group, the plasma CNO C_{max} values were 1399, 1932, and 3191 ng/ml after 3.0, 5.6, and 10.0 mg/kg, respectively. There was a trend-level, dose-dependent increase in the AUC with CNO-HCl [CNO-HCl AUC: $F(2,10) = 3.46$, $P = 0.07$], as well as increased variance between monkeys as dose increased (Fig. 1C; Table 3). For both CNO-DMSO and CNO-HCl groups, the S.D. of plasma CNO was over 50% of the mean at the highest doses, reflecting the high individual variability.

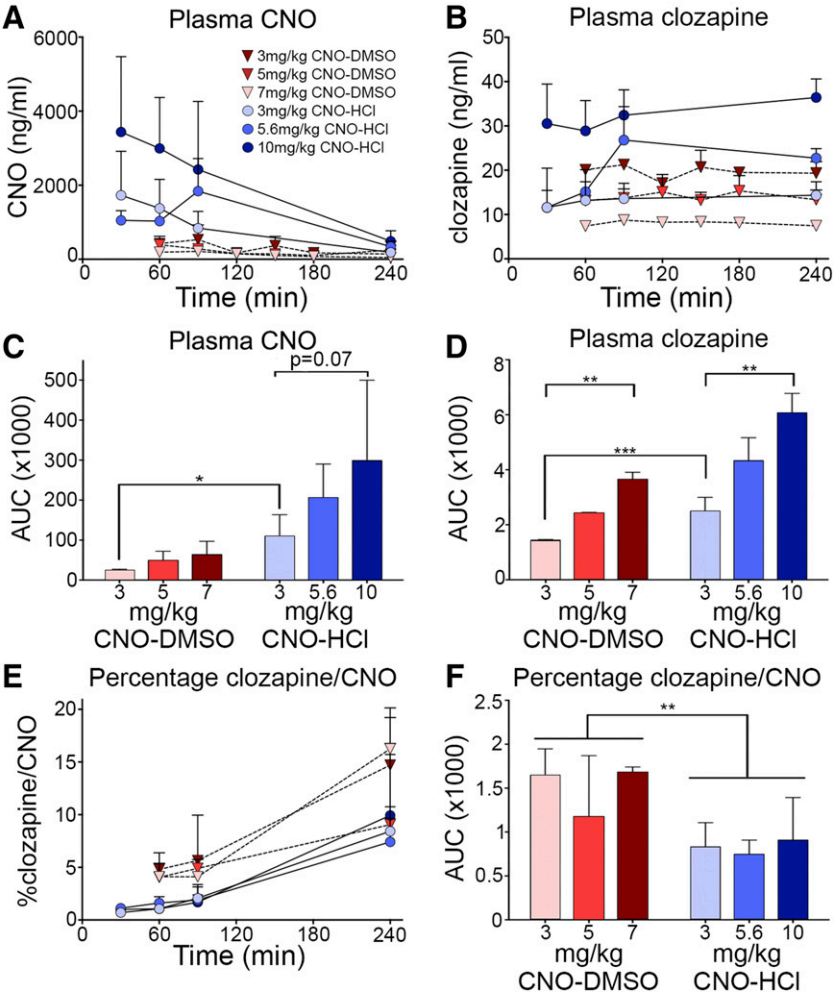

Fig. 1. Plasma pharmacokinetics of CNO-DMSO and CNO-HCl. (a and b) Four-hour time courses for plasma CNO (a) and clozapine (b) following intramuscular injection of CNO-DMSO (red colors, $n = 2$) and CNO-HCl (blue colors, $n = 6$). Doses are indicated in the inset (a). (c and d) Data from the 60, 90, and 240 time points were used to calculate the AUC values to directly compare plasma CNO (c) and clozapine (d) across drug dose and preparation. (e and f) The percentage of clozapine/CNO was calculated and presented over a 4-hour time course (e) and the collapsed AUC (f). All values for the AUC have been divided by a factor of 1000 (plotted AUC value of five corresponds to a true value of 5000). All data are presented as mean ± S.D. Statistical significance is denoted by the asterisks (*$P <$ 0.05; **$P < 0.01$; ***$P < 0.001$).

TABLE 3
Pharmacokinetic parameters following intramuscular injection of CNO-DMSO or CNO-HCl
Data are presented as mean ± S.D.

Drug/Dose	C_{max}[a]	T_{max}[a]	AUC_{60-240}[a]	Half-Life[b]	V_d (Observed)[c]	CL (Observed)[c]
mg/kg	*ng/ml*	*min*	*μg/ml·min*	*min*	*l*	*l/min*
Plasma CNO						
CNO-DMSO						
3.0	226 ± 20	75 ± 21	25 ± 2	82 ± 5	75 ± 29	0.6 ± 0.2
5.0	415 ± 197	75 ± 21	50 ± 22	1391 ± 1817	98 ± 37	0.4 ± 0.6
7.0	595 ± 308	75 ± 21	65 ± 32	137 ± 99	103 ± 92	0.5 ± 0.1
CNO-HCl						
3.0	1399 ± 754	65 ± 12	110 ± 53	69 ± 11	18 ± 12	0.2 ± 0.1
5.6	1932 ± 704	85 ± 12	207 ± 84	106 ± 55	25 ± 20	0.2 ± 0.0
10	3191 ± 1558	75 ± 16	300 ± 200	72 ± 24	20 ± 7	0.2 ± 0.1
Plasma clozapine						
CNO-DMSO						
3.0	8.7 ± 0.4	90 ± 0	1.5 ± 0.007	—	—	—
5.0	16.4 ± 2.1	165 ± 106	2.4 ± 0.014	—	—	—
7.0	22.6 ± 1.2	90 ± 0	3.7 ± 0.2	—	—	—
CNO-HCl						
3.0	15 ± 2.9	160 ± 88	2.5 ± 0.5	—	—	—
5.6	27.9 ± 5.7	140 ± 77	4.3 ± 0.8	—	—	—
10	39.0 ± 4.4	190 ± 77	6.1 ± 0.7	—	—	—
Clozapine:CNO (%)						
CNO-DMSO						
3.0	16.2 ± 3.9	240 ± 0	1.6 ± 0.3	—	—	—
5.0	9.4 ± 7.2	240 ± 0	1.2 ± 0.7	—	—	—
7.0	14.7 ± 4.5	240 ± 0	1.7 ± 0.06	—	—	—
CNO-HCl						
3.0	8.4 ± 2.3	240 ± 0	0.8 ± 0.3	—	—	—
5.6	7.4 ± 2.2	165 ± 106	0.8 ± 0.2	—	—	—
10	9.9 ± 5.8	240 ± 0	0.9 ± 0.5	—	—	—

CL, clearance; C_{max}, peak plasma concentration; T_{max}, time of peak plasma concentration; V_d, volume of distribution; —, V, CL, and half life were only calculated for the administered drug, CNO.
[a]Only data from sampling time points that were common to both forms of the drug were included in these calculations (60, 90, and 240 min postdrug administration).
[b]Terminal half-life was calculated using all three samples: 60, 90, and 240 min post-injection.
[c]The *V* and CL values were estimated from intramuscular injections based on the observed fraction of drug absorbed.

Plasma CNO concentrations between 60 and 240 minutes were used to calculate the terminal half-life, observed V_d, and observed clearance for both drug groups (Table 3). Overall, there were no significant dose effects on any of these parameters within either the CNO-DMSO or CNO-HCl groups. However, the volume of distribution was notably higher in the CNO-DMSO group compared with the CNO-HCl groups, with mean V_d values between 75-103 l compared with 18–25 l. Additionally, observed clearance was higher for the CNO-DMSO groups, ranging from 0.4 to 0.6 l/min compared with 0.2 l/min for all CNO-HCl doses tested.

Plasma clozapine concentrations (nanograms per milliliter) were dose dependent in both CNO-DMSO and CNO-HCl groups, as measured by AUC values across 60, 90, and 240 minutes post-injection [CNO-DMSO: $F(2,2) = 137.0$, $P = 0.007$; CNO-HCl: $F(2,10) = 31.2$, $P < 0.0001$] (Fig. 1D; Table 3). In the CNO-DMSO group, peak concentrations of clozapine were 8.7, 16.4, and 22.6 ng/ml following 3.0, 5.0, and 7.0 mg/kg, respectively (Fig. 1B). Clozapine variance was lower between monkeys than was observed for plasma CNO, with S.D. of less than 13% of the mean. For CNO-HCl, peak clozapine values were 15, 27.9, and 39.0 ng/ml for 3.0, 5.6, and 10.0 mg/kg, respectively, with S.D. up to 21% of the mean (Table 3).

Pharmacokinetic Comparison between CNO-DMSO and CNO-HCl in Plasma. The results from 3.0 mg/kg doses of CNO-DMSO and CNO-HCl were used for direct comparison between the two drug preparations. In general, CNO-HCl was associated with significantly higher plasma levels of CNO compared with CNO-DMSO [$t(5.03) = 3.91$, $P = 0.01$] and clozapine [$t(5.01) = 5.12$, $P = 0.004$] as measured by the AUC (Fig. 1, C and D). The difference in plasma CNO levels after peripheral administration of 3 mg/kg between the two drug preparations was nonoverlapping as well, with peak CNO concentrations in the CNO-DMSO group between 200 and 250 ng/ml compared with peak levels in the CNO-HCl group between 1000 and 4000 ng/ml (Fig. 1A). In the CNO-HCl group, half of the monkeys (3/6) had peak plasma CNO concentration at 30 minutes following drug administration. However, these values were not included in the statistical comparison since this time point was not available for the CNO-DMSO group. When looking at only common time points, peak CNO concentrations occurred at 60 minutes for both drug groups. As shown in Table 3, CNO-HCl resulted in plasma CNO levels between 540 and 2280 ng/ml, whereas CNO-DMSO resulted in plasma CNO between 200 and 215 ng/ml. Plasma clozapine levels at 60 minutes following drug administration for the CNO-DMSO group ranged from 8 to 9 ng/ml, and from 8 to 26 ng/ml for the CNO-HCl group (Fig. 1B). Thus, despite a 2- to 10-fold increase in plasma CNO measured in the CNO-HCl group, peak plasma clozapine increased only 3-fold (and five out of six monkeys had plasma clozapine concentrations below 15 ng/ml in the CNO-HCl group).

Relative Concentrations of Clozapine/CNO in Plasma Following CNO-DMSO and CNO-HCl Administration. Because these are within-sample assays, the percentage of clozapine/CNO may be a more accurate measure of

clozapine conversion (Fig. 1, E and F). The relative concentration of clozapine compared with CNO (percentage of clozapine/CNO) increased as a function of time post-administration in both CNO-DMSO and CNO-HCl preparations, consistent with decreasing CNO concentrations and relatively stable clozapine concentrations over time (Fig. 1, A, B, and E; Table 3). For both CNO-DMSO and CNO-HCl (within preparation dose-response comparisons), there was no effect of CNO dose on the ratio of clozapine/CNO as measured by collapsed AUC, indicating a relatively constant back-conversion of CNO to clozapine (Fig. 1F; CNO-DMSO AUC: $P = 0.39$; CNO-HCl AUC: $P = 0.66$). When comparing the two preparations, the ratio of clozapine/CNO was significantly higher in the CNO-DMSO group when collapsed across dose [AUC, $t(7.02) = 3.59$, $P = 0.009$] (Fig. 1F). At 60 minutes following CNO administration, the relative concentration of clozapine/CNO was nonoverlapping between the two groups, with CNO-DMSO samples ranging from 3.2% to 5.0% clozapine/CNO and CNO-HCl samples ranging from 0.6% to 1.5% clozapine/CNO.

Plasma Pharmacokinetics of Intravenous Administration of CNO-HCl. Route of administration (intramuscular and intravenous) was compared with plasma concentrations of CNO-HCl collected 30 minutes after 5.6 mg/kg CNO-HCl administration. CNO was modestly higher at 30 minutes after intravenous administration (mean of differences: 875 ng/ml), but this was only at trend level [$F(1,5) = 5.1$, $P = 0.07$] (Fig. 2A). Clozapine concentrations were not different between intramuscular and intravenous routes of administration, but showed a trend for lower concentrations following intravenous administration [$F(1,5) = 4.29$, $P = 0.09$] (Fig. 2B). The relative amount of clozapine to CNO (percentage of clozapine/CNO) was significantly lower in the plasma after intravenous compared with intramuscular administration [$F(1,5) = 18.29$, $P = 0.008$] (Fig. 2C).

CSF Pharmacokinetics of Intravenous Administration of CNO-DMSO and CNO-HCl. CNO-DMSO (6.5 mg/kg) and CNO-HCl (5.6 mg/kg) were both administered intravenously under sedation and CSF samples were collected between 30 and 60 minutes for CNO and clozapine assay. CSF concentrations of CNO in the CNO-DMSO group were between 17 and 21 ng/ml (mean 19 ng/ml, equivalent to 55 nM), while that of the CNO-HCl group were between 38 and 109 ng/ml (mean: 67 ng/ml, equivalent to 196 nM) (Fig. 3A). This effect was significant [$t(6.1) = 3.7$, $P = 0.01$], such that there was a higher concentration of CNO in the CSF in the CNO-HCl group, despite the lower dose administered. Despite higher concentrations of CNO following CNO-HCl, the CSF concentrations of clozapine appeared lower in these samples as well (not significant, $P = 0.26$) (Fig. 3B). CSF concentrations of clozapine were between 1.2 and 3.8 ng/ml (mean: 2.0 ng/ml, equivalent to 6.2 nM) following administration of CNO-DMSO (6.5 mg/kg, i.v.) and ranged from 0.4 to 0.9 ng/ml (mean: 0.6 ng/ml, equivalent to 1.9 nM) following CNO-HCl (5.6 mg/kg, i.v.) (Fig. 3B). The percentage of clozapine to CNO in the CSF in the CNO-DMSO group ranged from 5.6% to 22.5% (mean 11.2%), while the percentage of CSF clozapine to CNO in the CNO-HCl group ranged from 0.5% to 1.6% (mean: 1.0%) (Fig. 3C). The difference between CNO-DMSO and CNO-HCl for relative concentrations of clozapine/CNO was not statistically significant ($P = 0.2$).

Relative Amount of CNO in CSF and Plasma. To examine the distribution of CNO from the plasma to the CSF, a relative ratio of CNO measured in the CSF and plasma was calculated for all doses in which both samples were collected at a single time point (Fig. 4). For the intramuscular CNO-DMSO samples, there was no effect of CNO-DMSO dose on the percentage of CNO in the CSF/plasma [Fig. 4A; $F(2,2) = 2.5$, $P = 0.29$]. For the intravenous administrations of CNO-DMSO and CNO-HCl, there were also no differences in the ratio of CNO in the CSF/plasma (Fig. 4B; $t = 0.09$, $P = 0.9$). Across both drug preparations and routes of administrations tested, the relative amount of CNO in the CSF was between 1% and 7% of plasma CNO (Fig. 4, A and B).

Discussion

The data presented here represent the first report on the pharmacokinetics of a water-soluble salt form of CNO, as well as adds to the existing literature on CNO pharmacokinetics in the rhesus monkey (Eldridge et al., 2016; Nagai et al., 2016; Raper et al., 2017). The data show a clear increase in the solubility and bioavailability of CNO when prepared as a salt and dissolved in saline (CNO-HCl) compared with preparations using DMSO as a dissolvent in a saline solution (CNO-DMSO). From a methodological perspective, eliminating the need for DMSO is expected to reduce the discomfort of animal subjects and potential confounds with behavioral outcomes. In particular, although it was previously thought that DMSO concentrations of less than 10% could be well-tolerated, recent studies have shown that even at concentrations as low a 2%–4% (v/v), DMSO can induce apoptosis through inhibition of

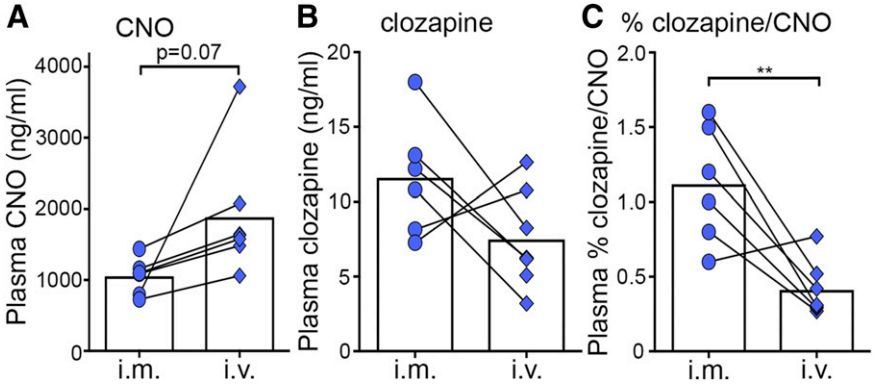

Fig. 2. Pharmacokinetics of intramuscular and intravenous routes of administration for CNO-HCl (5.6 mg/kg). (a–b) Plasma CNO (a), clozapine (b), and percentage of clozapine to CNO (c) following administration of 5.6 mg/kg CNO-HCl either intramuscularly or intravenously. All data points represent a single subject and bars represent group mean values. In a few cases, plasma concentrations were double determined for a single subject, in which case they were averaged before inclusion in this analysis. Intramuscular injections took place under awake conditions; intravenous injections were under 1% isofluorane anesthesia. Statistical significance is denoted by the asterisks (**$P < 0.01$).

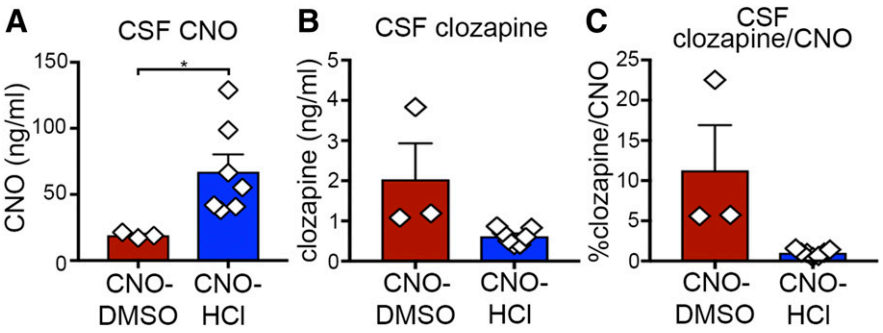

Fig. 3. CSF concentrations of CNO and clozapine following intravenous drug administration. CSF concentrations of CNO (a), clozapine (b), and the relative percentage of clozapine to CNO (c) 45–60 minutes after 6.5 mg/kg CNO-DMSO, i.v. (n = 3) or 30 minutes after 5.6 mg/kg CNO-HCl, i.v. (n = 7). Statistical significance is denoted by the asterisks (*$P < 0.05$).

mitochondrial respiration (Galvao et al., 2014). Additionally, DMSO has a long half-life of 16 hours in rhesus monkeys and is not eliminated fully for 72 hours after administration (Layman and Jacob, 1985), prolonging the toxic effects as well as limiting the frequency of repeated testing. This slow rate of elimination is in contrast to mice, which show almost complete elimination of DMSO by 8 hours after administration (Kaye et al., 1983).

The current data highlight several important factors that may improve our understanding of the absorption and distribution of CNO and clozapine. There are several common features of CNO-DMSO and CNO-HCl. First, the between-subject variability in the plasma concentrations of CNO was high, with S.D. up to 50% of the mean concentrations at the highest doses in both forms. This finding is similar to the clinical literature for clozapine, which reports large differences in plasma clozapine levels across patients given the same dosing regimen (Chang et al., 1998; Olesen, 1998; Chetty and Murray, 2007). For application to DREADD studies, this finding suggests the importance of determining the circulating concentration of CNO by time and dose in individual monkeys. Some of the variance may be explained by the rate and extent of the metabolism between CNO, clozapine, and other metabolites (Chang et al., 1998). The relative amount of plasma clozapine varied between monkeys and was not dose dependent, consistent with zero-order kinetics of this pathway through the cytochrome P450 system (Chang et al., 1998). Second, the relative time course of CNO and clozapine concentrations in plasma were consistent across both forms of the drug, such that plasma CNO levels peaked within 90 minutes but clozapine levels remained constant or slowly rising over the 4-hour sampling window, consistent with earlier reports in humans (Chang et al., 1998). Lastly, one feature common to both forms of CNO was that the ratio of CNO in the plasma to that in CSF was consistent across all doses and routes of administration (Fig. 4). This is particularly important for future DREADD research since it indicates that an approximate value of the CSF concentration can be reliably estimated from plasma CNO concentrations, providing further support to the importance of measuring plasma CNO when possible.

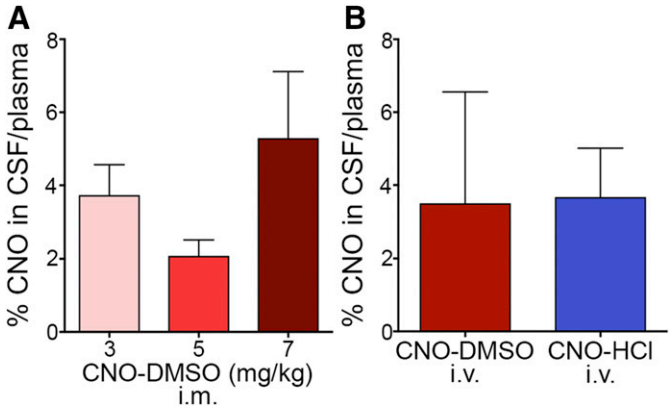

Fig. 4. Relative amount of CNO in CSF and plasma. (a) Percentage of CNO measured in CSF to plasma following intramuscular CNO-DMSO administration (3.0, 5.0, and 7.0 mg/kg) (b) Percent of CNO measured in the CSF to plasma following intravenous CNO-DMSO (6.5 mg/kg) and CNO-HCl (5.6 mg/kg) administration. Data are presented as mean ± S.D.

Beyond the similarities, there were also notable differences between CNO-DMSO and CNO-HCl. Most notably, the absolute value of plasma CNO concentrations were four to five times higher on average in the CNO-HCl group compared with the CNO-DMSO group when the same doses were administered (Fig. 1C; Table 3). While the mechanism of this difference is not yet known, it appears to occur during the absorption and distribution of CNO following intramuscular injection, as indicated by the greater V_d value in the CNO-DMSO group compared with the CNO-HCl samples. One hypothesis is that the presence of DMSO, while improving the solubility of CNO, is impairing the transport across biologic membranes from the muscle into the blood stream. However, the highly permissible structure of DMSO and ability to diffuse quickly and efficiently across compartments makes this somewhat unlikely (Rammler and Zaffaroni, 1967; Brayton, 1986). Additionally, the ratio of CNO in the CSF/plasma was consistent between CNO-DMSO and CNO-HCl groups (Fig. 4B), suggesting a similar distribution of CNO from the plasma to the brain. Another hypothesis is that the CNO-HCl solution is more stable. CNO-DMSO occasionally precipitated out of the solution, while the CNO-HCl remained in the solution for up to 8 hours. However, further analyses of the chemical properties of CNO-DMSO versus CNO-HCl are necessary, such as the stability at different pH levels, to fully characterize the pharmacokinetic profile in each of the distribution compartments.

The current study demonstrated low levels of clozapine found in both the plasma and CSF. The levels of clozapine reported in the CSF in the CNO-HCl group (Fig. 3B) were below the levels reported to activate muscarinic DREADDs in culture (Armbruster et al., 2007; Gomez et al., 2017). Apart from clozapine acting at the DREADD in addition to CNO, a primary concern with the presence of clozapine is that it will have off-target effects on any one of its known receptor targets,

including the D_1, D_2, D_4, α_1, α_2, 5-hydroxytryptamine (5-$HT)_{2A}$, 5-HT_{2B}, and 5-HT_3 receptors (Bymaster et al., 1996; Selent et al., 2008). Specifically, at 30 minutes when CNO concentrations are at peak levels, clozapine in the CSF was less than 3 nM for all subjects (<1 ng/ml; 3.08 conversion factor from nanograms per milliliter to nanomolars). Data from in vitro binding studies in rat brain tissue suggest higher clozapine binding affinities than 3 nM. In fact, a fast-cyclic voltammetry study in adult macaque slices suggests that concentrations of 1–100 nM of clozapine do not alter the concentration of dopamine (data not shown), in agreement with a previous study using the same technique that suggested clozapine concentrations over 300 nM are needed to affect dopamine levels (Bull and Sheehan, 1991). Specifically, clozapine affinity (K_i) for the D_4, 5-HT_{2A}, and 5-HT_{2C} receptors was in the 10–30 nM range, for D_1, D_2, and 5-HT_3 receptors in the 65–125 nM range, and for the 5-HT_{1A}, 5-HT_{1B}, and 5-HT_{1D} receptors in the 750–1200 nM range (Bymaster et al., 1996). While receptor-binding studies are informative, the translatability of these data to functional activity is limited. Most of the research on clozapine activity has come from serum levels of clozapine following treatment in schizophrenics. These studies commonly report a threshold level of 350–400 ng/ml for effectiveness of clozapine in most patients (Potkin et al., 1994; Olesen, 1998; Spina et al., 2000). However, more detailed analyses have identified some patients that show a response at "subthreshold" clozapine concentrations of approximately 150 ng/ml (Olesen, 1998). One study used positron emission topography to correlate plasma concentrations with receptor occupancy and found that plasma clozapine concentrations of 140 ng/ml (~430 nM) were associated with 80%–90% receptor occupancy at 5-HT_2 receptors (Nordström et al., 1995). In summary, much of the human subject data on plasma clozapine concentrations suggest only concentrations far exceeding the levels of clozapine reported in either plasma or CSF in this study to be of consequence.

It is essential to note the relatively high concentrations of CNO present in the CSF 30 minutes after drug administration (100–400 nM). Based on the time-response curves also presented (Fig. 1A; Table 3), it is expected that plasma CNO levels would continue to rise for another 30–60 minutes, leading to a similar increase in CSF concentrations of CNO. The absolute magnitude of CNO in the CSF is significantly improved in the CNO-HCl group, in comparison with the CNO-DMSO group here, as well as earlier reports with CNO in DMSO in rhesus monkeys (Raper et al., 2017). CNO concentrations >100 nM are expected to activate hM3D and hM4D DREADD receptors, leading to functional changes that can excite or inhibit cellular activity (Armbruster et al., 2007). Raper et al. (2017) also reported that CNO acts as a substrate for the efflux protein Pgp in cell culture, and suggested this mechanism was active in vivo to inhibit distribution across the blood-brain barrier. In contrast, our data show excellent brain penetration of CNO by increasing its solubility in saline. Furthermore, the relative amount of clozapine is greatly reduced, particularly within 2 hours after CNO administration when behavioral experiments typically occur. Given the findings of water-soluble CNO-HCl on animal compliance, brain availability, minimal back-metabolism to clozapine at concentrations that are below reported affinities for off-target receptor systems, and adaptation to behavioral experimental designs, it appears that CNO-HCl has significant advantages over CNO preparations in DMSO. Thus, CNO-HCl as a DREADD ligand continues to be a valuable tool in the understanding of functional brain circuitry in macaques.

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Authorship Contributions

Participated in research design: Allen, Grant, Cuzon Carlson.
Conducted experiments: Allen, Carlson.
Contributed new reagents or analytic tools: Xiong, Jin.
Performed data analysis: Allen.
Wrote or contributed to the writing of the manuscript: Allen, Carlson, Grant, Cuzon Carlson.

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