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Chronic behavioral manipulation via orally delivered chemogenetic actuator in macaques

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1 **Chronic behavioral manipulation via orally delivered**
2 **chemogenetic actuator in macaques**

3

4 Running title: Chronic chemogenetic control of monkey behavior

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6 Kei Oyama¹, Yukiko Hori¹, Yuji Nagai¹, Naohisa Miyakawa¹, Koki Mimura¹,
7 Toshiyuki Hirabayashi¹, Ken-ichi Inoue^{2,3}, Masahiko Takada², Makoto Higuchi¹,
8 Takafumi Minamimoto^{1*}

9

10 ¹ Department of Functional Brain Imaging, National Institutes for Quantum Science
11 and Technology

12 ²Systems Neuroscience Section, Primate Research Institute, Kyoto University,
13 Inuyama, Aichi 484-8506, Japan

14 ³PRESTO, Japan Science and Technology Agency, Kawaguchi, Saitama, Japan

15

16 * Author to whom all correspondence should be addressed:

17 Takafumi Minamimoto, Ph. D.

18 Department of Functional Brain Imaging, National Institutes for Quantum Science
19 and Technology

20 4-9-1 Anagawa, Inage-ku, Chiba 263-8555 Japan

21 Phone: +81-43-206-4699

22 Fax: +81-43-253-0396

23 E-mail: minamimoto.takafumi@qst.go.jp (T.M.)

24

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47

48 **Abstract**

49 The chemogenetic technology referred to as designer receptors exclusively
50 activated by designer drugs (DREADDs) offers reversible means to control
51 neuronal activity for investigating its functional correlation with behavioral action.
52 Deschloroclozapine (DCZ), a recently-developed highly potent and selective
53 DREADDs actuator, displays a capacity to expand the utility of DREADDs for
54 chronic manipulation without side-effects in nonhuman primates, which has not yet
55 been validated. Here we investigated the pharmacokinetics and behavioral effects
56 of orally administered DCZ in female and male macaque monkeys.
57 Pharmacokinetic analysis and positron emission tomography (PET) occupancy
58 examination demonstrated that oral administration of DCZ yielded slower and
59 prolonged kinetics, and that its bioavailability was 10-20% of that in the case of
60 systemic injection. Oral DCZ (300-1000 µg/kg) induced significant working memory
61 impairments for at least 4 h in monkeys with hM4Di expressed in the dorsolateral
62 prefrontal cortex (Brodmann's area 46). Repeated daily oral doses of DCZ
63 consistently caused similar impairments over two weeks without discernible
64 desensitization. Our results indicate that orally delivered DCZ affords a less
65 invasive strategy for chronic but reversible chemogenetic manipulation of neuronal
66 activity in nonhuman primates, and this has potential for clinical application.

67

68 **Significance Statement**

69 The use of DREADDs for chronic manipulation of neuronal activity for days to
70 weeks may be feasible for investigating brain functions and behavior on a long
71 time-scale, and thereby for developing therapeutics for brain disorders, such as
72 epilepsy. Here we performed pharmacokinetics and in vivo occupancy study of
73 orally administered DCZ to determine a dose range suitable for DREADDs studies.
74 In monkeys expressing hM4Di in the prefrontal cortex, single and repeated daily
75 doses significantly induced working-memory impairments for hours and over two
76 weeks, respectively, without discernible desensitization. These results indicate that
77 orally delivered DCZ produces long-term stable chemogenetic effects, and holds
78 great promise for the translational use of DREADDs technology.

79

80 **Introduction**

81 The chemogenetic tool referred to as DREADDs (designer receptors exclusively
82 activated by designer drugs) has been widely used for controlling neuronal activity
83 and animal behavior in many species, including rodents and nonhuman primates
84 (Roth, 2016). By combining mutated muscarinic receptors (excitatory hM3Dq and
85 inhibitory hM4Di) with specific actuators such as CNO (clozapine-N-oxide), these
86 chemogenetic techniques offer a means to remotely manipulate the activity of a
87 specific neuronal population for minutes to hours (Urban and Roth, 2015). In
88 addition to the acute control, repeated administration of the actuators enables
89 chronic chemogenetic manipulation of neuronal activity for days to weeks (Grace et
90 al., 2016; Nation et al., 2016; Wakaizumi et al., 2016; Andreoli et al., 2017; Yu and
91 Münzberg, 2018; Desloovere et al., 2019; Paretkar and Dimitrov, 2019; Goossens
92 et al., 2021), and thus has potential therapeutic applications. For example, chronic
93 and reversible inhibition of neuronal activity can be an effective means for seizure
94 control and epilepsy treatment (Lieb et al., 2019). For this purpose, oral delivery of
95 agonists seems to be suitable, as it typically produces relatively long-lasting effects
96 compared to systemic (i.e., intramuscular and intravenous) injections (Karbwan et
97 al., 1997). Besides, oral administration is non-invasive and relieves the subject
98 from stress during chronic treatment. It is also beneficial for neuroscience research
99 aimed at long-term control of neuronal activity in freely-moving animals without
100 restraint or stress. However, recent studies have reported that CNO has low
101 permeability into the brain, and that its metabolite, clozapine, activates not only

102 DREADDs but also endogenous receptors and transporters, leading to potential
103 off-target effects (Gomez et al., 2017; Tran et al., 2020). To circumvent this issue,
104 alternative selective DREADDs agonists have been explored and validated
105 (Bonaventura et al., 2019; Weston et al., 2019; Nagai et al., 2020).

106 Deschloroclozapine (DCZ) is one of the 3rd-generation agonists for
107 muscarinic DREADDs that represents high permeability into the brain and is highly
108 potent and selective to DREADDs, as compared to prior agonists such as CNO
109 and C21 (Nagai et al., 2020). Importantly, low doses of DCZ exert chemogenetic
110 effects with rapid onset that last several hours in both rodents and nonhuman
111 primates (Nagai et al., 2020). This indicates that DCZ is a promising chemogenetic
112 actuator that can be used for a wide variety of objectives (e.g., Hirabayashi et al.,
113 2021; Hori et al., 2021; Oyama et al., 2021). In particular, its superior efficacy and
114 brain permeability permit its use for oral administration. In fact, it has been
115 demonstrated that orally delivered DCZ effectively induces behavioral changes in
116 mice and marmosets (Krause et al., 2021; Mimura et al., 2021). However, the long-
117 term chemogenetic action of DCZ remains unclear, specifically in terms of the
118 duration of a single-dosage effect and the impact of repetitive doses over several
119 days to weeks. These will provide knowledge sufficient for designing chronic
120 chemogenetic experiments and, in addition, for developing potential clinical
121 therapeutic applications.

122 Here we investigated the pharmacokinetics and in vivo occupancy of orally
123 administered DCZ to determine a dose range suitable for DREADDs studies in

monkeys. We then demonstrated that oral DCZ administration induced a chemogenetic behavioral effect lasting at least more than 4 h in macaque monkeys with hM4Di expressed in the prefrontal cortex. We also found that repetitive daily administrations of DCZ yielded consistent behavioral effects over weeks without apparent signs of desensitization.

Materials and Methods

Subjects

A total of 12 macaque monkeys [10 Japanese (*Macaca fuscata*) and 2 Rhesus monkeys (*Macaca mulatta*); 10 males, 2 females; 2.8-8.0 kg; age 4-10 years at the beginning of experiments] were used (Table 1). All experimental procedures involving animals were carried out in accordance with the Guide for the Care and Use of Nonhuman primates in Neuroscience Research (The Japan Neuroscience Society; https://www.jnss.org/en/animal_primates) and were approved by the Animal Ethics Committee of the National Institutes for Quantum Science and Technology. The monkeys were kept in individual primate cages in an air-conditioned room (pair-housing environments are under preparation; see the preface of the above guideline). A standard diet, supplementary fruits/vegetables and a tablet of vitamin C (200 mg) were provided daily.

Viral vector production

145 Adeno-associated virus (AAV) vector expressing hM4Di (AAV1-hSyn-hM4Di-IRES-
146 AcGFP, 4.7×10^{13} particles/mL) was produced by helper-free triple transfection
147 procedure, which was purified by affinity chromatography (GE Healthcare,
148 Chicago, USA). Viral titer was determined by quantitative PCR using Taq-Man
149 technology (Life Technologies, Waltham, USA).

150

151 **Surgical procedures and viral vector injections**

152 Surgeries were performed under aseptic conditions in a fully equipped operating
153 suite. We monitored body temperature, heart rate, SpO₂ and tidal CO₂ throughout
154 all surgical procedures. Monkeys were immobilized by intramuscular (i.m.) injection
155 of ketamine (5-10 mg/kg) and xylazine (0.2-0.5 mg/kg) and intubated with an
156 endotracheal tube. Anesthesia was maintained with isoflurane (1-3%, to effect).
157 Before surgery, magnetic resonance (MR) imaging (7 tesla 400mm/SS system,
158 NIRS/KOBELCO/Brucker) and X-ray computed tomography (CT) scans
159 (Accuitomo170, J. MORITA CO., Kyoto, Japan) were performed under anesthesia
160 (continuous intravenous infusion of propofol 0.2-0.6 mg/kg/min). Overlay MR and
161 CT images were created using PMOD® image analysis software (PMOD
162 Technologies Ltd, Zurich, Switzerland) to estimate stereotaxic coordinates of target
163 brain structures. After surgery, prophylactic antibiotics and analgesics
164 (cefmetazole, 25-50 mg/kg/day; ketoprofen, 1-2 mg/kg/day; 7 days) were
165 administered.

166 One monkey (#237) received injections of AAV vector into one side of the
167 amygdala (2 μ L x 2 locations). The monkey underwent a surgical procedure to
168 open burr holes (~8-mm diameter) for the injection needle. Viruses were pressure-
169 injected using a 10- μ L Hamilton syringe (model 1701 RN, Hamilton) with a 30-
170 gauge injection needle and a fused silica capillary (Nagai et al., 2016). The
171 Hamilton syringe was mounted into a motorized microinjector (UMP3T-2, WPI) that
172 was held by a manipulator (model 1460, David Kopf) on the stereotaxic frame.
173 After the dura mater was opened about 3mm, the injection needle was inserted into
174 the brain and slowly moved down 2 mm beyond the target and then kept stationary
175 for 5 min, after which it was pulled up to the target location. The injection speed
176 was set at 0.25 μ L/min. After each injection, the needle remained in situ for 15 min
177 to minimize backflow along the needle.

178 Two monkeys (#229 and #245) received injections of AAV vector into the
179 bilateral prefrontal cortex (Brodmann's area 46) as described previously (Nagai et
180 al., 2020; Oyama et al., 2021). The frontal cortex was exposed by removing a bone
181 flap and reflecting the dura mater. Handheld injections at an oblique angle to the
182 brain surface were made under visual guidance through an operating microscope
183 (Leica M220, Leica Microsystems GmbH, Wetzlar, Germany). Nine tracks were
184 injected in each hemisphere located at the caudal tip (1 track) and along the dorsal
185 (4 tracks) and ventral (4 tracks) bank of the principal sulcus. Viral vectors were
186 injected at 3 to 5 μ L per track depending on the depth. A total of 35 to 44 μ L of viral
187 aliquots was injected into each hemisphere.

188

189 **PET imaging**

190 PET imaging was conducted as previously reported (Nagai et al., 2020). Briefly,
191 PET scans were performed using a microPET Focus 220 scanner (Siemens
192 Medical Solutions USA, Malvern, USA). Monkeys were immobilized by ketamine
193 (5-10 mg/kg) and xylazine (0.2-0.5 mg/kg) and then maintained under anesthetized
194 condition with isoflurane (1-3%) during all PET procedures. Transmission scans
195 were performed for about 20 min with a Ge-68 source. Emission scans were
196 acquired in 3D list mode with an energy window of 350–750 keV after intravenous
197 bolus injection of [^{11}C]DCZ (324.9-382.3 MBq). Emission data acquisition lasted for
198 90 min. To estimate the specific binding of [^{11}C]DCZ, regional binding potential
199 relative to nondisplaceable radioligand (BP_{ND}) was calculated by PMOD® with an
200 original multilinear reference tissue model (MRTMo)(Yan et al., 2021). PET scans
201 were conducted at 45 days after injection of vectors for the two monkeys (#229 and
202 #245) and for one monkey expressing hM4Di in the amygdala (#237) between 496
203 and 984 days, during which enough hM4Di expression for the occupancy study
204 was observed after its introduction.

205

206 **Drug administration**

207 DCZ (MedChemExpress HY-42110) was dissolved in 2.5% dimethyl sulfoxide
208 (DMSO, FUJIFILM Wako Pure Chemical Co.), aliquoted and stored at -30°C . For
209 systemic i.m. injection, this stock solution was diluted in saline to a final volume of

210 100 µg/kg, and injected intramuscularly. For systemic oral administration (per os,
211 p.o.), the DCZ stock solution was diluted in saline to a final volume of 100 µg/kg,
212 300 µg/kg, or 1,000 µg/kg. The solution was then injected into some pieces of
213 snack or diluted in drinking water, which were given to the monkeys. It typically
214 took 2-3 min for the monkeys to consume. After each administration, we confirmed
215 that there was no food in the monkeys' mouths. Fresh solution was prepared on
216 each day of usage.

217

218 **Occupancy study**

219 An occupancy study was performed using one monkey expressing hM4Di in the
220 amygdala (#237), because it is ideal for placing voxels of interest and measuring
221 tracer binding in a large volume of tissue expressing hM4Di and non-DREADD
222 control on the other side, as in previous studies (Nagai et al., 2016, 2020). PET
223 procedures were described above except for pretreatment of DCZ or vehicle.
224 Pretreatment of DCZ or vehicle p.o. via a gastric catheter was followed by
225 anesthesia and emission scans at 30 and 90 min, respectively. The baseline scan
226 data were obtained within 3 weeks from the day of PET scans for occupancy
227 measurement to minimize the effect of hM4Di expression level changes during this
228 period. To estimate the specific binding of [¹¹C]DCZ, BP_{ND} was calculated by
229 PMOD® with MRTMo. The volume of interest for the hM4Di-positive region was
230 defined as the area where the BP_{ND} value of [¹¹C]DCZ was higher than 0.5, while
231 that of the control region was placed at the corresponding contralateral side.

232 Estimates of fractional occupancy were calculated according to the previous
 233 study (Nagai et al., 2020) by the following equation:

$$234 \quad Occ = ({}^{hM4Di}BP_{BL} - {}^{hM4Di}BP_{PT}) / ({}^{hM4Di}BP_{BL} - {}^{CON}BP_{BL})$$

235 where ${}^{hM4Di}BP_{BL}$ and ${}^{CON}BP_{BL}$ indicate BP_{ND} at the hM₄Di-expressing amygdala
 236 region and contralateral control region under baseline conditions, respectively,
 237 while ${}^{hM4Di}BP_{PT}$ indicates BP_{ND} at the hM₄Di-expressing amygdala region under
 238 pretreatment condition. The relationship between occupancy (Occ) and agonist
 239 dose (D_{DCZ}) was modeled by the Hill equation

$$240 \quad Occ = D_{DCZ}^n / (D_{DCZ}^n + ED_{50}^n)$$

241 where ED_{50} and n indicate the agonist dose achieving 50% occupancy and the Hill
 242 coefficient, respectively.

243

244 **Pharmacokinetics analysis**

245 Three monkeys (#226, #248, #249) were used to assess the concentration of DCZ
 246 and its metabolites in plasma or cerebrospinal fluid (CSF). First, the monkeys were
 247 administered DCZ intramuscularly or orally (100 µg/kg for i.m.; 300 µg/kg for p.o.).
 248 Then they were immobilized by i.m. injection of ketamine (5-10 mg/kg) and xylazine
 249 (0.2-0.5 mg/kg) and intubated with an endotracheal tube. Anesthesia was
 250 maintained with isoflurane (1-3%, to effect). Then blood and CSF were collected at
 251 30, 60, 90, 120, 150, 180, 210, 240 min after DCZ administration, and on the next
 252 day, monkeys were again anesthetized, and the samples were collected at 24 h
 253 after DCZ administration. Blood was collected with a heparinized syringe and

254 plasma samples were obtained by centrifugation at 3,500 g for 15 min. All samples
255 were stocked at -80°C until analysis.

256 The pretreatment protocols for CSF and plasma samples were described
257 previously (Nagai et al., 2020). Quantification of DCZ, and its metabolites C21 and
258 DCZ-N-oxide, was performed by multiple reaction monitoring (MRM) using a
259 Shimadzu UHPLC LC-30AD system (Shimadzu Corp., Kyoto, Japan) coupled to a
260 tandem MS AB Sciex Qtrap 6500 system (AB Sciex LLC, Framingham, MA, USA).
261 The following MRM transitions (Q1/Q3) were used to monitor each compound:
262 DCZ (293.0/236.0), C21 (279.0/193.0), DCZ-N-oxide (309.0/192.9). Other
263 protocols were described previously.

264 A blood sample was also collected from one monkey (#245) following oral
265 administration of 300 µg/kg of DCZ along with the daily behavioral testing under
266 awake conditions to quantify the concentration of DCZ. The collected sample was
267 treated and analyzed as well as those described above.

268

269 **Behavioral task**

270 Three monkeys (#226, #229, #245) were tested with a spatial delayed
271 response task. The protocol was almost the same as in our previous study (Oyama
272 et al., 2021). Behavioral testing was conducted in a sound-attenuated room.
273 Monkeys sat on a monkey chair from which they could reach out one hand and
274 take food to their mouths. A wooden table with two food-wells was placed in front of
275 the monkeys, and a screen was placed between the monkeys and the table. First,

276 a piece of food reward (raisin or snack) was placed in one of the two food-wells,
277 and then both wells were covered with wooden plates. Then, the screen was
278 placed for 0.5, 10 or 30 s, which served as delay periods. The position of the baited
279 well (left or right) and the delay period were determined pseudo-randomly. After the
280 delay period, the screen was removed and the monkeys were allowed to select
281 either food-well. The monkeys were allowed to get the food if they reached for the
282 correct food-well and removed the cover plate. The inter-trial interval was set at 10
283 s. A daily session lasted about one hour, and consisted of 3 blocks of 30 trials for
284 monkey #229, and 2 blocks of 30 trials for monkeys #226 and #245, which were
285 interleaved with 5-min rest periods. Behavioral tests began immediately or 0.5, 1, 3,
286 or 24 h after vehicle or DCZ treatment.

287 Another monkey without AAV injection (non-DREADD; #239) was tested with
288 a reward-size task using the same protocol as applied in a previous study
289 (Minamimoto et al., 2009). The behavioral testing began 1 h after an oral
290 administration of either vehicle or DCZ.

291

292 **Statistics**

293 To examine the effect of each treatment on the performance of the delayed
294 response task, behavioral measurement (correct rates) was subjected to Welch's t-
295 test or two-way ANOVA using GraphPad Prism 7.

296

297 **Histology and immunostaining**

298 For histological inspection, two monkeys (#229, #245) were deeply anesthetized
299 with an overdose of sodium pentobarbital (80 mg/kg, i.v.) and transcardially
300 perfused with saline at 4°C, followed by 4% paraformaldehyde in 0.1 M phosphate
301 buffered saline (PBS), pH 7.4. The brain was removed from the skull, postfixed in
302 the same fresh fixative overnight, saturated with 30% sucrose in phosphate buffer
303 (PB) at 4°C, and then cut serially into 50- μ m-thick sections on a freezing
304 microtome. For visualization of immunoreactive signals of GFP (co-expressed with
305 hM4Di), a series of every 6th section was immersed in 1% skim milk for 1 h at
306 room temperature and incubated overnight at 4°C with rabbit anti-GFP monoclonal
307 antibody (1:200-500, G10362, Thermo Fisher Scientific) in PBS containing 0.1%
308 Triton X-100 and 1% normal goat serum for 2 days at 4°C. The sections were then
309 incubated in the same fresh medium containing biotinylated goat anti-rabbit IgG
310 antibody (1:1,000; Jackson ImmunoResearch, West Grove, PA, USA) for 2 h at
311 room temperature, followed by avidin-biotin-peroxidase complex (ABC Elite, Vector
312 Laboratories, Burlingame, CA, USA) for 2 h at room temperature. For visualization
313 of the antigen, the sections were reacted in 0.05 M Tris-HCl buffer (pH 7.6)
314 containing 0.04% diaminobenzidine (DAB), 0.04% NiCl₂, and 0.003% H₂O₂. The
315 sections were mounted on gelatin-coated glass slides, air-dried, and cover-slipped.
316 A part of other sections was Nissl-stained with 1% Cresyl violet. Images of sections
317 were digitally captured using an optical microscope equipped with a high-grade
318 charge-coupled device (CCD) camera (Biorevo, Keyence, Osaka, Japan).

319 Results

320 *Pharmacokinetics of orally administered DCZ*

321 We first conducted pharmacokinetic studies in three naïve monkeys to estimate the
322 time course of the chemogenetic effects of DCZ via oral administration. In our
323 previous study, intramuscular administration of DCZ at 100 µg/kg provided a
324 sufficient level of DCZ concentration in CSF to activate hM4Di receptors for 2 h,
325 during which it gave rise to behavioral deficits in monkeys via hM4Di activation
326 (Nagai et al., 2020). Given that oral administration of clozapine, an analogue of
327 DCZ, results in relatively lower bioavailability and slower onset of action compared
328 to intravenous administration (Sun and Lau, 2000), we first examined DCZ
329 concentrations in the plasma and CSF for 4 h following oral DCZ administration
330 (300 µg/kg), as well as intramuscularly (100 µg/kg) as reference. Oral DCZ
331 administration yielded a maximum DCZ concentration in plasma at 60 min after
332 administration, followed by a gradual decrease (Fig. 1A, orange). By contrast,
333 intramuscular administration increased plasma DCZ concentration at 30 min, which
334 then decreased monotonically (Fig. 1A, green). Both oral and intramuscular
335 administrations produced relatively stable DCZ concentrations in CSF for at least 4
336 h (>2 nM and >5 nM; Fig. 1B, orange and green, respectively), which were
337 sufficient concentrations to activate hM4Di DREADD (EC₅₀ of 0.081 nM) (Nagai et
338 al., 2020). DCZ was not detected in plasma or CSF 24 h after administration. We
339 also examined the concentration of possible metabolites of DCZ, C21 and DCZ-N-
340 oxide in plasma; however, metabolites were not detected (Fig. 1A, light blue and

341 purple), confirming the metabolic stability of DCZ (Nagai et al., 2020). Taken
342 together, these results suggest that the oral administration of DCZ produces a
343 relatively long-lasting pharmacokinetic profile without significant metabolite
344 production and a lower bioavailability, about 10–20% of that of intramuscular
345 administration.

346

347 ***Measuring in vivo occupancy of hM4Di-DREADD by oral DCZ administration***

348 Next, to determine an effective and safe dose range for oral DCZ administration,
349 we measured hM4Di receptor occupancy by DCZ — the relationship between DCZ
350 dose and the degree of hM4Di receptor occupation (Fig. 2A). This was important
351 because a higher agonist dose will afford a stronger chemogenetic effect, but may
352 risk higher off-target effects (Upright and Baxter, 2020). Normally, proportional
353 receptor occupancy is a generalizable measure because it reflects the relationship
354 between target drug concentration and its receptor affinity, irrespective of brain
355 region or receptor density (Cunningham et al., 2010; Takano et al., 2016). In our
356 previous study, a series of PET studies was used to determine the optimal DCZ
357 dose that would yield a 50–80% occupancy of hM4Di, which was 30–100 µg/kg for
358 systemic administration (Fig. 2C, see Nagai et al., 2020 for details). Given that the
359 bioavailability of oral administration would be 10–20% of that of systemic
360 administration (Fig. 1), we estimated that oral DCZ doses achieving 50% and 80%
361 hM4Di occupancy would be around 300 and 1,000 µg/kg, respectively. We

362 performed a PET occupancy study using a monkey that had received injection of a
363 viral vector encoding an hM4Di gene into the unilateral amygdala (see Methods).

364 Baseline PET imaging with radiolabeled DCZ ($[^{11}\text{C}]\text{-DCZ}$) confirmed that the
365 hM4Di expression at the injected side of the amygdala was indicated by an
366 increased radioligand binding (Fig 2B, baseline). Subsequent $[^{11}\text{C}]\text{-DCZ}$ PET scans
367 were performed after oral pretreatments (i.e., oral administration of vehicle, 100,
368 300, or 1,000 $\mu\text{g/kg}$ of non-radiolabeled DCZ). Consistent with our previous
369 findings, specific binding of $[^{11}\text{C}]\text{DCZ}$ at the hM4Di-expressing amygdala region
370 was diminished with increasing doses of DCZ (Fig. 2B). Occupancy was
371 determined as a reduction of specific radioligand binding (BP_{ND}) at the target
372 region over the control side relative to baseline (Fig. 2B, see Materials & Methods
373 for details). As we predicted, the hM4Di occupancies with 100, 300 and 1,000
374 $\mu\text{g/kg}$ oral administration were about 35%, 50% and 70%, respectively (Fig. 2C).
375 The dose for 50% occupancy (EC_{50}) was 254 $\mu\text{g/kg}$, which corresponded to about
376 a 10-fold dose of systemic administration and was roughly consistent with the
377 bioavailable profile shown in our pharmacokinetic study described above. Based
378 on these results in pharmacokinetic and occupancy studies, we determined the
379 effective and safety dose range for oral DCZ administration to be 300–1,000 $\mu\text{g/kg}$.

380

381 ***Oral DCZ administration selectively induces longer-lasting behavioral effects***
382 ***in hM4Di-expressing monkeys***

383 We next examined the efficacy and time-course of the chemogenetic effect of oral
 384 DCZ administration using hM4Di-expressing monkeys. We used two monkeys with
 385 hM4Di expressed in the bilateral dorsolateral prefrontal cortex (dlPFC) around the
 386 principal sulcus (Brodmann's area 46; Fig. 3A,B), a brain region known to be
 387 responsible for spatial working memory and executive function (Fuster, 2015).
 388 Several weeks after the injections, we performed PET imaging with [^{11}C]DCZ to
 389 visualize hM4Di expression non-invasively. High levels of [^{11}C]DCZ binding ($\text{BP}_{\text{ND}} >$
 390 1.0) were localized in the bilateral dlPFC in both monkeys, specifically from the
 391 caudal end of the principal sulcus to 5-6 mm anterior to it (Fig. 3C,F). These PET
 392 signals reflected hM4Di expression, as post-mortem analysis confirmed GFP-
 393 positive zone in dorsal and ventral bank of the principal sulcus (Fig. 3D-H). When
 394 we compared the time course of [^{11}C]DCZ uptake in the whole brain of these
 395 monkeys with that of other monkeys that had not undergone surgery (Fig. 3I), there
 396 was no marked difference across the subjects. This suggested that the brain
 397 permeability of DCZ was unaffected by the dlPFC surgery.

398 Our previous studies have shown that chemogenetic silencing of the dlPFC
 399 via intramuscular DCZ administration (100 $\mu\text{g}/\text{kg}$) in these monkeys impaired
 400 performance in the spatial delayed response task (Fig. 4A) (Nagai et al., 2020;
 401 Oyama et al., 2021). Prior to the introduction of hM4Di, intramuscular DCZ
 402 administration (100 $\mu\text{g}/\text{kg}$) *per se* did not have an impact on working memory in
 403 #245 (Fig. 4B; Nagai et al., 2020), suggesting that oral administration of DCZ below
 404 1,000 $\mu\text{g}/\text{kg}$ would be expected not to have any side-effects. Then we directly

405 compared the effects of oral administration with those of intramuscular injections by
406 using the same subjects as in our previous studies that showed similar behavioraral
407 deficits (Nagai et al., 2020), which would enable us to demonstrate that oral DCZ
408 administration can be adapted to dlPFC, one of the core brain regions specialized
409 for primates. We examined the behavioral effects of two oral DCZ doses (300 and
410 1,000 $\mu\text{g/kg}$) and an intramuscular DCZ dose (100 $\mu\text{g/kg}$) for comparison.
411 Consistent with previous studies (Nagai et al., 2020; Oyama et al., 2021),
412 intramuscular 100 $\mu\text{g/kg}$ DCZ administration resulted in a delay-dependent
413 decrease in the task performance for 1-2 h following administration (Fig. 4E). Oral
414 DCZ administration of 300 and 1,000 $\mu\text{g/kg}$ DCZ also induced a comparable
415 impairment of the task performance for 1-2 h after administration (Figs. 4C and D).
416 We further assessed the time-course of the chemogenetic effects by conducting a
417 behavioral examination at 5 different time windows after treatment: 0–0.5, 0.5–1,
418 1–2, 3–4, and 24–25 h. We focused on the performance of the trials with the longest
419 delay period, where the effects were most prominent (Figs. 4C-E, 30s). In the first
420 30-min period, intramuscular administration significantly decreased the correct
421 rates (Fig. 4H, green) compared to those after vehicle administration (Fig. 4H,
422 cyan), indicating rapid behavioral effects as previously demonstrated (Nagai et al.,
423 2020). Significant effects were also observed following oral administration of 300
424 and 1,000 $\mu\text{g/kg}$ (Figs. 4F, G, orange and magenta, respectively). Similarly, all
425 treatments had significant effects at 0.5–1 and 1–2 h after administration (Fig. 4F-
426 H). At 3–4 h after administration, intramuscular administration had no influence on

427 performance (Fig. 4H), while the oral administrations remained effective (Figs. 4F,
428 G).

429 To determine the minimum effective dose of DCZ via oral administration, we
430 also administered 100 $\mu\text{g/kg}$ DCZ orally and examined the monkeys 1–2 and 3–4 h
431 thereafter. The performance was significantly impaired after 1–2 h (Welch's t-test, t
432 = 4.2, $p = 0.002$), but not after 3–4 h ($t = 0.48$, $p = 0.65$), indicating that this dose is
433 effective only for a shorter duration compared to 300 and 1,000 $\mu\text{g/kg}$.

434 Importantly, the decrease in task performance was found to be dependent
435 on delay duration (Fig. 4C), suggesting that the orally administered DCZ resulted in
436 loss of dlPFC function, but did not produce side-effects on other functions, such as
437 general attention or motivation. To further confirm that oral administration had no
438 unwanted side effects, we conducted control experiments with the delayed
439 response task (Fig. 5A) and a simple instrumental task (Fig. 5B, C) in two monkeys
440 to whom DREADDs had not been introduced (non-DREADD monkeys). Similar to
441 our previous study that tested with 100 $\mu\text{g/kg}$ of intramuscular DCZ administration
442 (Nagai et al., 2020), oral DCZ administration of 1,000 $\mu\text{g/kg}$ did not produce any
443 discernible behavioral changes in either task (Fig. 5A, C), suggesting that oral DCZ
444 administration itself is behaviorally inert at a dose of 1,000 $\mu\text{g/kg}$ or less.

445 Together, these results suggest that oral DCZ administration can induce
446 behavioral effects selectively in hM4Di-expressing monkeys for hours without
447 adverse side effects; specifically, a dose of 300–1,000 $\mu\text{g/kg}$ is effective for at least
448 4 h.

449

450 ***Chronic DCZ administration enables successive induction of chemogenetic***
451 ***silencing for weeks***

452 Finally, we investigated the efficacy of chronic DCZ administration across weeks.
453 One monkey expressing hM4Di in the dlPFC was tested daily with the delayed
454 response task to examine the chemogenetic effect of repetitive oral DCZ
455 administration. Chronic oral DCZ administrations for at least 2 weeks were
456 preceded and followed by several days of control vehicle administration (Fig. 6A,
457 B), and repeated twice with a sufficient interval of several months. This design
458 allowed us to investigate whether repetitive DCZ dose would consistently induce
459 behavioral deficits for weeks and how soon task performance would be recovered
460 after withdrawal of DCZ. Daily oral DCZ administrations (300 µg/kg) consistently
461 induced impairment of the performance of the delayed response task during 1–2 h
462 post-administration compared to that of following vehicle injections throughout the
463 repetitive oral DCZ period (Fig. 6C), indicating that apparent desensitization did not
464 occur. Previous studies have shown that chronic chemogenetic manipulation
465 caused posttreatment effects, such as rebound excitability (Desloovere et al., 2019;
466 Goossens et al., 2021), suggesting that it could induce some physiological
467 changes lasting even after agonist withdrawal. That was not the case in the current
468 study: we found that performance recovered to a normal level on the first day after
469 withdrawing from chronic DCZ administration (Fig. 6C), indicating that successive

470 behavioral deficits were the outcome of temporal chemogenetic silencing rather
471 than due to physiological change such as DREADD-induced plasticity.

472 It has been shown that plasma concentrations of the DCZ analogue
473 clozapine vary within subjects over weeks of treatment (Centorrino et al., 1994),
474 especially dropping by an average of more than 50% in the first 12 weeks (Kurz et
475 al., 1998). We confirmed that the concentration of DCZ following daily
476 administration was maintained at a similar level throughout the period of DCZ
477 administration, indicating that chronic manipulation did not alter the
478 pharmacokinetics of DCZ for at least 2 weeks (Fig. 6D). We also verified that
479 chronic DCZ administration for a single schedule (16 days for DCZ, and 7 and 3
480 days of vehicle administration, see Fig. 6A) alone had no significant impact on
481 spontaneous movements in a non-DREADD monkey (#253; Welch's t-test, $t =$
482 0.55 , $p = 0.59$). Taken together, these results suggested that DCZ is suitable for a
483 chronic and reversible manipulation of neuronal activity, leading to potential
484 therapeutic applications in the future.

485

486

487 **Discussion**

488 Here we demonstrated that oral administration of DCZ is an effective and non-
489 invasive means for manipulating neuronal activity through hM4Di receptors in
490 macaque monkeys. Our pharmacokinetics and *in vivo* PET occupancy examination
491 showed that the bioavailability of orally administered DCZ was about 10-20% of
492 that of intramuscular injection, and thus the appropriate dosage of oral DCZ to
493 exert hM4Di-mediated chemogenetic effects was estimated to be 300–1,000 µg/kg.
494 Indeed, this oral DCZ at this range of doses caused severe impairments in working
495 memory performance for several hours in monkeys with hM4Di expressed in
496 dlPFC, but did not induce any discernible behavioral side-effects in control non-
497 DREADD-expressing monkeys. Furthermore, repetitive daily oral DCZ doses over
498 two weeks yielded consistent chemogenetic effects without apparent signs of
499 desensitization. Taken together, the oral administration of DCZ affords a minimally
500 invasive strategy for chronic and reversible chemogenetic control of neuronal
501 activity via muscarinic DREADDs in nonhuman primates, thereby providing a great
502 potential for its clinical application.

503 For DREADDs studies in rodents and nonhuman primates, systemic
504 (intraperitoneal, intramuscular, or intravenous) injections are the standard
505 approach of agonist administration, as they allow precise control of dosage and
506 timing. However, such approach forces the animals to be restrained, which may
507 cause stress responses and unwanted effects on behavioral actions. Several
508 DREADDs studies have demonstrated that oral administration seems effective for

509 overcoming this issue. For example, oral CNO administration induced changes in
510 ethanol consumption by inhibition of the nucleus accumbens via hM4Di, in which
511 neuronal activity was remotely manipulated while the animals were unrestrained
512 (Cassataro et al., 2014). This is beneficial when wanting to examine the effect on
513 natural behavior including social communication (Zou et al., 2016). Oral CNO
514 administration has also been applied to manipulate the activity of non-neuronal
515 cells such as microglia (Grace et al., 2016). However, recent reports have revealed
516 that the effects of CNO are partly mediated by its back-conversion to clozapine
517 which crosses the blood-brain barrier and acts as a DREADDs actuator (Gomez et
518 al., 2017). Oral administration of low doses of clozapine and olanzapine as
519 agonists has also been attempted (Goossens et al., 2021). Due to their high
520 affinities for endogenous receptors, however, possible side effects are always a
521 matter of concern that cannot completely be ruled out.

522 In the current study, we evaded these issues by using DCZ, a novel
523 DREADDs agonist with high brain permeability and high selectivity. Since the
524 effects of drugs on the central nervous system depend on multiple factors such as
525 brain permeability, drug kinetics, and affinity for target receptors, it is generally
526 difficult to predict the optimal drug dosage. As we have demonstrated in previous
527 studies (Nagai et al., 2016, 2020), PET occupancy measurements together with
528 pharmacokinetics analysis are extremely effective ways for seeking the adequate
529 agonist dose range. We found that the bioavailability of orally administered DCZ
530 was 10-20% compared to systemic injections (see Figs. 1 and 2). Based on this

531 estimation, the dose range suitable for hM4Di activation was determined to be 300-
532 1,000 µg/kg. In line with the kinetics data, such oral DCZ doses induced relatively
533 long-lasting effects compared to intramuscular injections. The DCZ kinetics and
534 behavioral effects were examined up to 4 h after oral administration, during which
535 the concentration of DCZ persisted in CSF, and thus the chemogenetic effects may
536 be maintained longer.

537 The working-memory impairment following DCZ administration cannot be
538 explained by side-effects due to action on endogenous receptors as indicated by
539 other prior DREADDs agonists for the following reasons. First, one of the two
540 monkeys (#245) had been tested with the delayed response task before
541 introducing hM4Di in the dlPFC, showing that DCZ alone had no impact on the
542 behavior (Fig. 4B). Second, control experiments using non-DREADD expressing
543 monkeys confirmed that these oral DCZ doses alone did not produce any
544 discernible changes in behavior associated with spatial working memory or reward
545 expectation (Fig. 5). Third, PET and histological data suggest that two monkeys
546 consistently express hM4Di, covering the similar region of dlPFC. Collectively, we
547 concluded that behavioral results consistently observed in these two monkeys were
548 due to the inactivation of dlPFC neurons via hM4Di activation.

549 In the present study, we did not examine the effects of oral DCZ
550 administration on hM3Dq, an excitatory muscarinic DREADDs that responds to
551 DCZ (Nagai et al., 2020). Our previous work showed that the lower doses (1-3
552 µg/kg) of DCZ through intramuscular injection were capable of inducing significant

553 neuronal excitation in mice and monkeys expressing hM3Dq (Nagai et al., 2020).
554 Thus, 3-30 $\mu\text{g/kg}$ would be an effective range for activating hM3Dq via oral
555 administration. Indeed, we have recently demonstrated that marmosets expressing
556 hM3Dq produced consistent behavioral changes when fed a diet containing 10
557 $\mu\text{g/kg}$ of DCZ (Mimura et al., 2021). In any case, it should be noted that the optimal
558 agonist dose generally depends on the level of the overexpressed functional
559 protein (Upright et al., 2018) and the targeted circuit to be manipulated. In addition,
560 as is usual in studies using genetic methods, control experiments (e.g., using non-
561 DREADDs animals) are recommended.

562 It has been shown that chronic administration of DREADDs agonists
563 induces DREADDs-mediated changes for days to weeks in rodents (Nation et al.,
564 2016; Desloovere et al., 2019; Paretkar and Dimitrov, 2019; Goossens et al.,
565 2021). However, some studies reported that the effects following administrations of
566 agonists were not consistent during treatments. For example, Goossens et al.
567 reported that the administrations of clozapine and olanzapine caused significant
568 behavioral changes for several days, but that such effects subsequently diminished
569 even though the agonist administrations continued (Goossens et al., 2021). In
570 addition, it has been reported that inhibition by hM4Di with agonist treatment over
571 weeks induced posttreatment rebound excitability (Desloovere et al., 2019;
572 Goossens et al., 2021). In contrast to these studies, we showed that daily oral DCZ
573 delivery constantly impaired the monkey's performance throughout the
574 administration periods (see Fig. 6). Moreover, performance on the day following

575 completion of repeated doses of DCZ was as high as the baseline, suggesting that
576 weeks-long inactivation of the PFC did not affect subsequent behavior. These
577 results suggest that chronic DCZ administration did not cause desensitization of
578 hM4Di or any long-lasting change, such as plasticity of neural circuits that govern
579 working memory. It remains to be clarified whether this is a general capability of
580 DREADD/DCZ or is due to the characteristics of neurons and local circuits in the
581 dlPFC as a target. Irrespective of the mechanism, the present results indicate that
582 it is possible to investigate the effects of chronic attenuation of PFC activity on
583 various cognitive functions. Importantly, it can resolve potential discrepancies that
584 may arise in the behavioral outcomes of different durations or methods of
585 inactivation. For example, it has been demonstrated that acute and chronic
586 silencing of PFC interneurons had opposite impacts on anxiety-like behavior in
587 mice (Soumier and Sibille, 2014), suggesting that short- vs. long-term
588 manipulations of local circuits may differentially alter PFC network functions.
589 Besides, long-term abnormalities or imbalances in prefrontal activity have been
590 suggested as a pathophysiological mechanism for psychiatric disorders, since
591 chronic stress exposure could lead to anxiety disorder and depression (Zhou et al.,
592 2019). DREADDs subserve to mimic or reverse such long-term circuitry changes
593 that are beyond the reach of conventional acute blockade and/or irreversible
594 lesioning. The chemogenetic approach using DCZ as an actuator introduced in the
595 present study will expand the opportunity of acute and chronic manipulations of
596 specific circuits in the primate prefrontal cortex, thereby leading to a better

597 understanding of higher brain functions, e.g., working memory, decision-making,
598 and action-planning, in health and disease conditions. This can be done without
599 causing pain/stress to monkeys, and thus is also suitable for quantifying the effects
600 on unrestrained naturalistic, social behavior (e.g., using a markerless motion
601 tracking system; Bala et al., 2020; Labuguen et al., 2021). In addition, it has the
602 potential to be used in therapeutic applications for symptoms caused by abnormal
603 neuronal activity in a specific brain region, such as seizures in epilepsy.

604 Finally, it should be noted that this study did not examine continuous
605 chemogenetic effects beyond the duration of action of a single oral dose (~4 h).
606 Continuous silencing may be achieved with more frequent and repeated
607 administrations. It should also be noted that the utility of chronic oral administration
608 and the effect of DCZ in pre-DREADD condition were examined in a single monkey
609 (#245). Future studies will need to determine the dosage and interval of
610 administrations to maintain sufficient DCZ concentration to attain continuous
611 chemogenetic effects, and to ensure that it does not cause undesirable side
612 effects, toxicity or tachyphylaxis, with appropriate within-subject control using non-
613 DREADD animals.

614 DREADDs technology is becoming increasingly popular as a means of
615 controlling neuronal activity remotely, less-invasively and reproducibly in rodents
616 and nonhuman primates. With the accumulation of numerous successful reports,
617 muscarinic DREADDs are now under consideration for clinical application. Given
618 the long-term stable chemogenetic effects with orally delivered DCZ in macaque

619 monkeys, it holds great promise for the translational use of DREADDs technology,
620 especially in the development of therapeutic trials for neurological and
621 neuropsychiatric disorders.
622

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761

762

763

764 **Figure Legends**

765

766 **Figure 1.** Time concentration profiles of DCZ by oral and intramuscular
767 administration. **A**, Time-course of DCZ concentration in plasma (**A**) and CSF (**B**)
768 following intramuscular injection of 100 µg/kg (green) and oral administration of
769 300 µg/kg (orange) DCZ. Concentration of major metabolites of DCZ (DCZ-n-oxide
770 and C21, light blue and purple, respectively) following oral administration of 300
771 µg/kg DCZ are also shown (data for C21 were behind those of DCZ-n-oxide as
772 neither were detected). Data were collected from 3 monkeys (#226, #248, and
773 #249). Error bars represent s.e.m.

774

775 **Figure 2. PET visualizes the occupancy of orally administered DCZ for hM4Di.**

776 **A**, Schematic illustrations of occupancy study by PET. Monkeys underwent

777 [^{11}C]DCZ-PET scan 90 min after p.o. administration of nonradiolabeled DCZ or

778 vehicle. **B**, Coronal sections of parametric [^{11}C]DCZ-PET image of specific binding

779 (BP_{ND}) overlaying MR image of a monkey expressing hM4Di in the amygdala.

780 Scale bars represent 10 mm. **C**, Occupancy of hM4Di plotted as a function of DCZ.

781 Red triangles represent occupancy by oral DCZ doses obtained from monkey

782 #237. Curves are the best-fit Hill equation for the data. ED_{50} indicates the agonist

783 dose inducing 50% occupancy. Hill coefficient (n) and coefficient of determination

784 (R^2) values are as follows: p.o. $n = 0.66$, $R^2 = 0.988$; i.v.: $n = 1$, $R^2 = 0.99$. Note that

785 the data for intravenous (i.v.) injection (gray) refers to a previous study (Nagai et

786 al., 2020).

787

788 **Figure 3. Introduction of hM4Di in bilateral dlPFC.**

789 **A**, Illustration showing the injection sites for AAV vector carrying an hM4Di gene.
790 The left panel presents a zoomed-in view of the framed area of the right panel. **B**,
791 A coronal plane corresponding to the lines in the lateral view. Red dots and shaded
792 area represent intended injection sites. **C,F**, In vivo visualization of hM4Di
793 expression in dlPFC for #229 (**C**) and #245 (**F**). Coronal PET image showing
794 specific binding of [¹¹C]DCZ overlaying MR images. **D,G**, Corresponding nissl-
795 stained sections for #229 (**D**) and #245 (**G**). **E,H**, Corresponding DAB-stained
796 sections representing immunoreactivity against a reporter protein for #229 (**E**) and
797 #245 (**H**). Open and filled arrowheads represent the dorsal and ventral borders of
798 the target regions, respectively. Scale bars, 5mm. **I**, The time course of whole-brain
799 uptake of [¹¹C]DCZ for 7 monkeys including #229 and #245. Shaded area
800 represents the standard deviation of 5 monkeys excluding #229 and #245. Please
801 note that #229 and #245 were the same subjects as used in our previous study
802 (Oyama et al., 2021). SUV, standardized uptake value [regional radioactivity
803 (Bq cm^{-3}) $\times$ body weight (g)/injected radioactivity (Bq)].

804 **Figure 4. Effects of oral and intramuscular administration of DCZ on a**
 805 **cognitive task in monkeys expressing hM4Di in the dIPFC.**
 806 **A**, Delayed response task. **B**, Effects of intramuscular administration of 100 µg/kg
 807 of DCZ on task performance for monkey #245 before (“Pre-vector”) and after
 808 (“Post-vector”) introduction of hM4Di (Nagai et al., 2020). Two-way ANOVA
 809 (treatment × delay) revealed significant main effects of treatment after introduction
 810 of hM4Di (treatment; $F_{(1,24)} = 188$, $p = 7.3 \times 10^{-13}$; delay; $F_{(2,24)} = 57.3$, $p = 7.3 \times 10^{-10}$;
 811 interaction; $F_{(2,24)} = 10.9$, $p = 4.3 \times 10^{-4}$), but not before its introduction
 812 (treatment; $F_{(1,24)} = 0.03$, $p = 0.86$; delay; $F_{(2,24)} = 19.9$, $p = 8.0 \times 10^{-7}$; interaction;
 813 $F_{(2,24)} = 1.4$, $p = 0.26$). **C-E**, Effects of oral administration of 300 µg/kg (orange) and
 814 1,000 µg/kg (magenta), and intramuscular administration of 100 µg/kg (green) of
 815 DCZ on task performance. The same data for the vehicle were plotted in each
 816 panel (cyan). The behavioral tests were conducted 1-2 h following each
 817 administration. In all conditions, two-way ANOVA (treatment × delay) revealed
 818 significant main effects of treatment and delay, and interaction (100 µg/kg, i.m.;
 819 treatment: $F_{(1,30)} = 17.4$, $p = 2.3 \times 10^{-4}$; delay: $F_{(2,30)} = 41.6$, $p = 2.2 \times 10^{-9}$;
 820 interaction: $F_{(2,30)} = 13.5$, $p = 6.4 \times 10^{-5}$; 300 µg/kg, p.o.; treatment; $F_{(1,30)} = 60.9$, p
 821 $= 1.1 \times 10^{-8}$; delay; $F_{(2,30)} = 117.1$, $p = 7.0 \times 10^{-14}$; interaction; $F_{(2,30)} = 40.5$, $p = 3.0$
 822 $\times 10^{-9}$; 1000 µg/kg, p.o.; treatment; $F_{(1,30)} = 22.9$, $p = 4.2 \times 10^{-5}$; delay; $F_{(2,30)} = 62.7$,
 823 $p = 1.9 \times 10^{-11}$; interaction; $F_{(2,30)} = 21.4$, $p = 1.7 \times 10^{-6}$), indicating the delay-
 824 dependent behavioral deficits. The data from two monkeys were pooled (N = 6
 825 sessions; 3 sessions for each monkey). **F-H**, Time-course of behavioral effects of

826 each administration. Only the correct rates of 30-s trials were plotted, as they were
827 most prominent, as shown in (C-E). Asterisks indicate significant differences
828 compared to vehicle administrations ($p < 0.05$, Welch's t-test, uncorrected). Error
829 bars represent s.e.m.
830

831 **Figure 5. Effects of 1000 µg/kg DCZ oral administration on behavioral tasks in**
832 **non-DREADD animals. A,** Performance of delayed response task following oral
833 administration of vehicle (cyan) and 1000 µg/kg DCZ (red) in a non-DREADD
834 animal (#226). There was no significant difference between treatments (two-way
835 ANOVA, treatment × delay, treatment: $F(1,24) = 0$, $p = 1$). **B,** Illustration of a
836 reward-size task. Each trial in this task began when the monkey touched a lever,
837 which was followed by the appearance of a visual cue signaling the size of the
838 upcoming reward (1, 2, 4 or 8 drops). To obtain the reward, the monkeys had to
839 release the lever when a visual target changed color from red to green. **C,** Example
840 behavioral data of reward size task for DCZ (1000 µg/kg, p.o.; red) and vehicle
841 administration session (cyan) obtained from a non-DREADD monkey (#239). Two-
842 way ANOVA (treatment × reward size) revealed no significant difference ($p > 0.05$).
843 Error bars represent s.e.m.
844

845 **Figure 6. Chronic behavioral effect of daily DCZ administration across weeks.**

846 **A**, Schedule of experiments. In a series of sessions, after 7 days of vehicle
 847 administrations (Veh), DCZ was orally administered for 16 days, followed by 3 days
 848 of vehicle, once each day. The monkey (#245) was tested by delayed response
 849 task over 5, 12 and 3 days, respectively. The series of testing were repeated twice.
 850 Cyan and purple circles represent the drugs administered (vehicle or DCZ), and the
 851 character “T” below the circles represents the days on which the behavioral tests
 852 were conducted. **B**, Schedule of an experiment in one day. **C**, Correct rates plotted
 853 as a function of days for vehicle (cyan) and DCZ (red) administrations for the 1st
 854 (circle) and 2nd (triangle) series, respectively. There was significant difference
 855 between vehicle and DCZ administrations (two-way repeated measures ANOVA
 856 with treatment and schedule (1st and 2nd schedules), main effect of treatment,
 857 $F_{(1,18)} = 194$, $p = 4.4 \times 10^{-11}$). There was no significant difference between
 858 performance during the former and latter periods (two-way repeated measures
 859 ANOVA with timing (former vs latter) and schedule, main effect of timing, $F_{(1,10)} =$
 860 0.03 , $p = 0.87$), indicating that the impairment was consistent during the period of
 861 administration. **D**, Plasma DCZ concentration plotted as a function of days for
 862 vehicle (cyan) and DCZ (red) administrations for the same monkey (#245). Data
 863 were collected as with the behavioral data in (**D**), that is, 1 h after oral
 864 administration of 300 $\mu\text{g/kg}$ DCZ. Note that the DCZ concentration appeared to be
 865 relatively higher than those of other monkeys in Fig. 1A. This might be attributable
 866 to the difference of sampling conditions, i.e., under anesthesia or awake conditions

867 (see Methods for details). It is also possible that oral administration of DCZ may be
868 less efficient for crossing the blood-brain barrier in animal #245, since the PET data
869 suggests that brain uptake of DCZ in #245 was relatively low compared to the other
870 animals (although it was still within a standard range; Fig. 3I).

871 **Table 1. Summary of monkeys used in the study.**

872

ID	Species	Sex	Age (y)	Vector	Occupancy	PK	DCZ uptake	Behavior
201	R	M	10	hM4Di OFC			✓	
221	J	M	7	hM4Di OFC			✓	
226	J	F	8	N/A		✓		✓
228	R	M	7	N/A			✓	
229	R	M	5	hM4Di dIPFC			✓	✓
237	J	M	7	hM4Di amygdala	✓			
239	J	M	6	N/A				✓
240	J	M	6	hM4Di OFC			✓	
245	J	F	7	hM4Di dIPFC		✓	✓	✓
248	J	M	4	N/A		✓		
249	J	M	4	N/A		✓		
253	J	M	5	N/A				✓
Total	12 (R,2/J,10)	F,2; M,10		6	1	4	6	5

873

874 A tick indicates the subject was used in the experiment. PK, pharmacokinetics
875 study; OFC, orbitofrontal cortex; Species (J, Japanese; R, Rhesus), Sex (F,
876 Female; M, Male), Age (years). Note that monkeys #229 and #245 were used in
877 our previous studies (Nagai et al., 2020; Oyama et al., 2021).

878

879

A

B

C