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Selective activation of cholecystokinin-expressing γ -aminobutyric acid (CCK-GABA) neurons enhances memory and cognition

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1 Manuscript Title Page

2 **1. Manuscript Title:** Selective activation of cholecystokinin-expressing γ -aminobutyric acid
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15 PW, JB, IK, MG and GP conducted behavioral testing. JK and JB did immunohistochemistry.
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17 helped develop the experimental approach and contributed valuable experimental reagents.

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43 **Title**

44 Selective activation of cholecystokinin-expressing γ -aminobutyric acid (CCK-GABA)
45 neurons enhances memory and cognition

46 **Abstract**

47 Cholecystokinin-expressing GABAergic (CCK-GABA) neurons are perisomatic inhibitory
48 cells that have been argued to regulate emotion and sculpt the network oscillations
49 associated with cognition. However, no study has selectively manipulated CCK-GABA
50 neuron activity during behavior in freely-moving animals. To explore the behavioural
51 effects of activating CCK-GABA neurons on emotion and cognition, we utilized a novel
52 intersectional genetic mouse model coupled with a chemogenetic approach.
53 Specifically, we generated triple transgenic *CCK-Cre;Dlx5/6-Flpe;RC::FL-hM3Dq* (CCK-
54 GABA/hM3Dq) mice that expressed the synthetic excitatory hM3Dq receptor in CCK-
55 GABA neurons. Results showed that CNO-mediated activation of CCK-GABA neurons
56 did not alter open field or tail suspension performance and only slightly increased
57 anxiety in the elevated plus maze. Though CNO treatment had only modestly affected
58 emotional behavior, it significantly enhanced multiple cognitive and memory behaviors
59 including social recognition, contextual fear conditioning, contextual discrimination,
60 object recognition and problem-solving in the puzzle box. Collectively, these findings
61 suggest that systemic activation of CCK-GABA neurons minimally affects emotion but
62 significantly enhances cognition and memory. Our results imply that CCK-GABA
63 neurons are more functionally diverse than originally expected and could serve as a
64 potential therapeutic target for the treatment of cognitive/memory disorders.

65 **Significance Statement**

66 CCK-GABA neurons are thought to play an important role in pathologies such as
67 schizophrenia, but their contributions to behavior in the healthy states are poorly
68 understood. Here we report a novel method for selectively targeting CCK-GABA
69 neurons and manipulating their activity during behavioural tasks. Our data demonstrate

70 that activating CCK-GABA neurons subtly affects emotional behavior but surprisingly
71 enhances multiple memory and cognitive processes.

72 **Introduction**

73 The inhibitory neurotransmitter γ -aminobutyric acid (GABA) critically regulates
74 information processing by modifying neuronal excitability and synaptic plasticity
75 (Wigstrom and Gustafsson, 1983; Sloviter, 1991). GABAergic transmission contributes
76 to multiple behaviors, including anxiety, fear, pain, memory, olfaction and social
77 interaction (Enna and McCarson, 2006; Makkar et al., 2010; Schmidt et al., 2014).
78 Dysregulation of GABAergic transmission is a defining feature of many pathologies,
79 including schizophrenia, chronic pain, depression and Alzheimer's disease (Rissman et
80 al., 2003; Enna and McCarson, 2006; Luscher et al., 2011; Taylor and Tso, 2015).
81 GABAergic signaling is orchestrated by interneurons, a heterogenous cell population
82 composed of many subtypes differing in morphological, electrophysiological and
83 neurochemical properties as well as connectivity patterns (Kepecs and Fishell, 2014).
84 There is widely believed to be a 'division of labor' among these many interneurons,
85 wherein distinct subtypes are specialized for certain functions (Kepecs and Fishell,
86 2014).

87 Interneurons expressing the neuropeptide cholecystokinin (CCK; termed CCK-
88 GABA neurons) are recognized for their characteristic axonal projections, which ramify
89 extensively around the perisomatic regions (i.e. cell body, proximal dendrites, and axon
90 initial segments) of post-synaptic pyramidal cells. It has been hypothesized that CCK-
91 GABA neurons are functionally specialized for the regulation of emotional behaviors
92 such as mood, anxiety and fear (Freund, 2003). Notably, CCK-GABA neurons express
93 several receptor subtypes involved in controlling emotional behavior, including 5-
94 hydroxytryptamine type 3 receptors (5-HT₃) and cannabinoid type 1 (CB1) receptors
95 (Morales and Bloom, 1997; Marsicano and Lutz, 1999). Additionally, the post-synaptic
96 targets of CCK-GABA neurons are enriched with α 2-containing GABA_A receptors, which
97 regulate anxiety behaviors and the effects of anxiolytic drugs (Freund, 2003). However,
98 compelling evidence for the role of CCK-GABA neurons in emotional behaviors has

99 proven elusive, with studies producing conflicting findings (Truitt et al., 2009; Brown et
100 al., 2014; Schmidt et al., 2014; Bowers and Ressler, 2015).

101 An alternative and underappreciated possibility is that CCK-GABA cells play an
102 influential role in cognitive and memory processes. Several lines of evidence support
103 this theory. Firstly, CCK-GABA cells are comparatively abundant (20 – 30% of all GABA
104 neurons) in brain regions involved in cognitive/memory processes, such as the medial
105 prefrontal cortex, hippocampus and ventrolateral temporal cortex (Whissell et al., 2015).
106 Second, CCK-GABA neurons are thought to orchestrate the theta frequency network
107 oscillations associated with cognitive processes as they elicit slow, asynchronous
108 inhibitory events in their targets (Curley and Lewis, 2012; Nagode et al., 2014). Third,
109 the presynaptic nerve terminals of CCK-GABA neurons are enriched with CB1 receptors
110 (Katona et al., 1999), which have been implicated in cognition and memory as well as
111 the neuroplasticity that accompanies these processes (Carlson et al., 2002).
112 Collectively, these remarkable features suggest CCK-GABA neurons are well-
113 positioned to regulate cognition and memory.

114 To gain better insight into the behavioral effects of enhanced CCK-GABA neuron
115 activity, we selectively activated these cells during tests of emotion, cognition and
116 memory by using an intersectional genetic approach (Sciolino et al., 2016) coupled with
117 the chemogenetic technique (Armbruster et al., 2007). In our novel transgenic line, the
118 CCK-GABA/hM3Dq mouse, CCK-GABA neurons express the synthetic excitatory Gq-
119 coupled hM3Dq receptor which can be selectively activated by the synthetic ligand,
120 clozapine-N-oxide (CNO). To determine the behavioural effects of CCK-GABA neuron
121 activation, we studied different emotional and cognitive behaviors in CCK-GABA/hM3Dq
122 mice given CNO or vehicle injection. We observed that CCK-GABA neuron activation
123 minimally affected emotional behavior but significantly enhanced memory and cognition.

124

125 **Materials and Methods**

126 *Animals.* To generate triple transgenic *CCK-Cre;Dlx5/6-Flpe;RC::FL-hM3Dq* mice
 127 (termed CCK-GABA/hM3Dq⁺ mice), homozygous CCK-ires-Cre mice (C57BL/6 genetic
 128 background, B6N.Cg-*Cck*^{tm1.1(cre)Zjh}/J, JAX#019021) were first crossed with homozygous
 129 *RC::FL-hM3Dq* mice (C57BL/6 genetic background) (Sciolino et al., 2016).
 130 Subsequently, double transgenic *CCK-Cre;RC::FL-hM3Dq* mice were crossed with
 131 *Dlx5/Dlx6-FLPe* mice (FVB/NC genetic background, Tg(mI56i-FLPe)39Fsh/J,
 132 JAX#010815) to obtain triple transgenic mice having all three alleles (Cre, hM3Dq and
 133 Flpe). Double transgenic mice having Cre and hM3Dq alleles but not the Flpe allele
 134 were referred to as CCK-GABA/hM3Dq⁻ mice and used as age-matched littermate
 135 controls. Both male and female mice were used in experiments. Mice were group
 136 housed with *ad libitum* access to food and water in a temperature-controlled room on a
 137 12 h light/dark cycle. All behavioral testing occurred during the light phase. Animals
 138 underwent multiple behavioral tests in the following order (starting with the least
 139 aversive test and proceeding to the most aversive test): open field test, elevated plus
 140 maze test, novel object recognition test, puzzle box test, social interaction test and fear
 141 conditioning test. As the fear conditioning test resulted in long-term changes in animal
 142 behavior, a separate population of naïve animals was used for the tail suspension test.
 143 In all behavioral assays but the novel object recognition test, a between-subject design
 144 was used wherein animals were randomly assigned to either the vehicle injection
 145 condition or drug injection condition before the experiment began. In all branches of the
 146 novel object recognition test, which followed a within-subject design, animals were
 147 tested twice and received each injection. Experimental procedures were in accordance
 148 with the guidelines of the Canadian Council on Animal Care (CCAC) and the local
 149 Animal Care Committee at [Author Affiliation].

150 *Immunohistochemistry and image acquisition.* Triple transgenic mice 3-6 months old
 151 were anesthetized with avertin and underwent transcardial perfusion with 0.1 M
 152 phosphate buffered saline (PBS; pH 7.4) followed by 4% paraformaldehyde (PFA) in
 153 PBS. Extracted brains were placed into 4% PFA at 4°C for 24h and then transferred into
 154 a PBS solution containing 30% sucrose at 4°C for 48h. Afterwards, brains were cut into

155 40 μ M sections using a cryostat (CM1520; Leica) maintained at -20°C. From each brain,
 156 10 sections were obtained in the area of the dorsal hippocampus (Bregma = -1.34 to -
 157 1.94 mm).

158 In wide field microscopy experiments, tissue sections were rinsed with 0.1 M
 159 PBS and blocked with 5% normal donkey serum in 0.1% Triton-X-100 PBS (PBS-T) for
 160 1 h at room temperature. Sections were then incubated with chicken polyclonal anti-
 161 GFP (1: 1000; ab13970; Abcam, Cambridge, MA, USA) and rabbit polyclonal anti-
 162 mCherry (1:1000; ab167453; Abcam) primary antibodies in PBS-T for 48 hr at 4 °C.
 163 Thereafter, sections were rinsed with PBS-T and incubated with Alexa 488-conjugated
 164 donkey anti-chicken (1:1000; 703545145; Jackson ImmunoResearch; West Grove, PA,
 165 USA) and Alexa 594-conjugated donkey anti-rabbit (1:1000; 715515152; Jackson
 166 ImmunoResearch) secondary antibodies in PBS-T for 2 hr at room temperature.
 167 Sections were then rinsed with PBS-T and mounted on Superfrost Plus slides (Fisher
 168 Scientific, Pittsburgh, PA, USA) and coverslipped with Aquamount (Polysciences Inc.,
 169 Warrington, PA, USA). In experiments examining colocalization of mCherry with CCK
 170 and GAD67, sections were stained using a different procedure. During primary antibody
 171 staining, sections were incubated with goat anti-mCherry antibody (1:1000; AB0040;
 172 Sicgen; Cantanhede, Portugal), mouse anti-GAD67 antibody (1:1000; Mab5406;
 173 Milipore; Billerica, MA, USA) and rabbit polyclonal anti-CCK-8 antibody (1:1000; C2581;
 174 Sigma Aldrich; St. Louis, MO, USA) in PBS-T for 48 hr at 4 °C. In secondary antibody
 175 staining, sections were incubated in PBS-T with Alexa 594-conjugated donkey anti-goat
 176 antibody (1:1000; 705515147; Jackson ImmunoResearch), DyLight 488-conjugated
 177 donkey anti-mouse antibody (1:1000; 715485150; Jackson ImmunoResearch) and
 178 Alexa 405- conjugated donkey anti-rabbit antibody (1:1000; ab175651; Abcam) for 2 hr
 179 at room temperature.

180 For cell counting experiments, images of brain sections were generated using an
 181 FSX100 fluorescent microscope (Olympus). Alexa 488 and 594 signals were captured
 182 using an U-MWIBA3 filter cube (Ex460-495, Em510-550, DM505) for Alexa 488 and an
 183 U-MWIG3 filter cube (Ex530-550, Em575IF, DM570) for Alexa 594. In acquired images,
 184 regions of interest in the hippocampus and prefrontal cortex were delineated manually

185 according to area definitions established by Paxinos and Franklin (2012). Automated
 186 cell counting of GFP- and mCherry-labeled cells in delineated brain regions was
 187 performed using cellSens 1.7 software (Olympus). For each animal, we calculated the
 188 relative abundance of mCherry-labelled cells for a region of interest (= mCherry-labelled
 189 cells/[mCherry-labelled cells + GFP-labelled cells]) by averaging all values for that
 190 region across all sections. For colocalization experiments, images were captured
 191 through a Quorum spinning disk confocal microscope (Zeiss) using a 20x objective lens
 192 and were analyzed with Volocity Software (Perkin Elmer). Alexa Fluor 405, 488, and
 193 594 (secondary antibody signals) were excited with the 405 nm, 491 nm and 561 nm
 194 laser, respectively.

195 *Drugs.* Clozapine-N-oxide (CNO) was obtained from the NIH as a part of the Rapid
 196 Access to Investigative Drug Program funded by the National Institute of Neurological
 197 Disorders and Stroke (NINDS). CNO powder was dissolved in 20% dimethylsulfoxide
 198 (DMSO)/saline to prepare a stock solution of 3 mM. In behavioral experiments, mice
 199 were weighed daily before being randomly assigned to either CNO or vehicle treatment
 200 groups. Unless otherwise stated, all drug injections were given in the intraperitoneal
 201 cavity approximately 10 min prior to each test. Mice in the CNO group received a 3
 202 mg/kg injection of CNO whereas mice in the vehicle group received a DMSO/saline
 203 injection. The experimenters giving the injections and testing the mice were blinded to
 204 the drug condition. To ensure that CNO injection of an animal in one behavioral test did
 205 not confound the performance of that animal in another subsequent behavioral test, we
 206 made sure that different behavioral tests were separated by at least 48h. In
 207 electrophysiology experiments, a concentration of 5 μ M CNO was used.

208 *Electrophysiology - Tissue Preparation.* Male CCK-GABA/hM3Dq⁺ and CCK-
 209 GABA/hM3Dq⁻ mice 2-3 months old were used. Mice were deeply anesthetized with
 210 chloral hydrate (400 mg/kg i.p.) followed by transcardial perfusion with ice-cold solution
 211 containing (in mM): 50 sucrose, 92 NaCl, 15 D-Glucose, 26 NaHCO₃, 5 KCl, 1.25
 212 NaH₂PO₄, 0.5 CaCl₂, 7 MgSO₄, 1 kynurenic acid that was oxygenated with 95% O₂/5%
 213 CO₂. The brain was removed and transverse hippocampus slices (300 μ m) were cut
 214 with a vibratome (Leica VT-1200S) and thereafter incubated at room temperature in

215 artificial cerebrospinal fluid (aCSF) containing (in mM): 124 NaCl, 3 KCl, 1.25 NaH₂PO₄,
 216 1.3 MgCl₂, 2.6 CaCl₂, 26 NaHCO₃ and 10 D-glucose (300 to 310 mOsm) that was
 217 oxygenated with 95% O₂/5% CO₂.

218 *Electrophysiology - Recording.* Hippocampal slices were perfused with aCSF at 2-3
 219 ml/min. Cells were visually identified using a microscope (BX-51W1; Olympus, Center
 220 Valley, PA, USA) fitted with IR-DIC and X-Cite LED120 fluorescence illumination
 221 (Excelitas Technologies, Waltham, MA, USA) filtered with FITC and TRITC cubes
 222 (Olympus) for detection of mCherry expression in CCK-GABA/hM3Dq⁺ cells. The
 223 recording pipette had a resistance of 4 – 6 MΩ. For current-clamp experiments the
 224 pipette was filled with intracellular solution containing (in mM): 132.5 K-gluconate, 17.5
 225 KCl, 10 HEPES, 0.2 EGTA, 2 Mg-ATP and 0.3 GTP (pH 7.25, 290 mOsm). For voltage-
 226 clamp experiments where mIPSCs were recorded the intracellular solution contained (in
 227 mM): 140 CsCl, 10 HEPES, 11 EGTA, 1 CaCl₂, 2 MgCl₂, 2 tetraethylammonium
 228 chloride, 4 Mg-ATP (pH 7.25, 290 mOsm). Neurons were held at -60mV, and mIPSCs
 229 were recorded with TTX (0.2 μM), APV (50 μM) and CNQX (10 μM) added to the aCSF.
 230 Spontaneous action potential frequency and resting membrane potential were recorded
 231 in current clamp mode. TTX (0.2 μM) was added to the aCSF for determination of
 232 resting membrane potential. Electrophysiological recordings were amplified with
 233 Multiclamp 700A (Molecular Devices), filtered at 2 kHz, sampled 50 kHz, and analyzed
 234 offline with Clampfit 10 (Molecular Devices). Stable 3-min recordings of mIPSCs were
 235 selected for measuring mIPSC amplitude and frequency. Action potential frequency was
 236 measured by averaging the number of spontaneous action potentials over a 5-min
 237 window at baseline or after bath application of CNO.

238 *Open field test.* Animals were placed in an open field box (50 cm L × 50 cm W × 20 cm
 239 H) for 10 min. The 35 × 35 region in the center of the arena was defined as the center
 240 whereas the remainder was defined as the periphery zone. Automated scoring of zone
 241 time and distance travelled was coordinated by an experimenter blind to condition using
 242 Ethovision XT 11.5 software.

243 *Elevated plus maze.* The 5 minute test was conducted in a plus-shaped maze
 244 composed of two “open” arms without walls (20 L × 10 W cm) and two “closed” arms (20

245 L × 10 W cm) enclosed by walls (10 cm H) arranged around a center zone (10 cm L ×
 246 10 cm W). Automated scoring of arm time, arm entries and distance travelled was
 247 coordinated by an experimenter blind to condition using Ethovision XT 11.5 software.

248 *Tail Suspension test.* The test took place in a tall chamber (20 cm L × 50 cm W × 50 cm
 249 H). To prevent bending of the mouse's tail during the test, a lightweight plastic cylinder
 250 (8 cm long, 2 g in weight) was placed around the tail base. Next, a length of adhesive
 251 tape (20 cm) was gently but firmly wrapped around the tail 2 cm from the tail tip. This
 252 length of tape was then fixed to the roof of the chamber, suspending the mouse in the
 253 air. The test lasted 6 minutes and the time that the mouse spent immobile was scored
 254 by an experimenter blind to condition.

255 *Social interaction test.* The 3-chambered sociability test was conducted under red light
 256 (3 lux) in a clear Plexiglas box with three compartments (40 cm L × 20 cm W × 40 cm H)
 257 separated by walls. The center-facing walls of the left and right chambers contained an
 258 opening (5 cm W × 40 cm H) that allowed passage into the center chamber. Each of the
 259 left and right chambers contained a wire cage (11 cm D × 11 cm H, 1 cm bar spacing).
 260 Prior to testing, mice were habituated to the apparatus for 10 min (Habituation trial).
 261 Afterward, the test mouse was placed in a holding cage while a novel juvenile mouse
 262 (23 - 35 d of age) unfamiliar to the test mouse was placed inside one of the wire cages.
 263 The test mouse was returned to the apparatus and the time spent interacting with the
 264 stranger mouse was recorded (Sociability trial, 10 minutes). Afterward, both mice were
 265 removed and placed in separate holding cages while the apparatus was cleaned with
 266 70% ethanol. The mouse from the Sociability trial – now familiar to the test mouse –
 267 was then returned to one of the wire cages. A second novel mouse, completely
 268 unfamiliar to the test mouse, was then added in the opposite wire cage (Social
 269 recognition trial, 10 minutes). The test mouse was then returned to the apparatus and
 270 interaction time with the 'familiar' and 'novel' mice was measured manually by an
 271 experimenter blind to condition using a keystroke function in Ethovision XT 11.5
 272 software.

273 *Fear conditioning and contextual fear discrimination.* A conditioning chamber (20 cm L ×
 274 20 cm W × 30 cm H) with a shock grid floor consisting of stainless steel bars (2 cm

275 apart, diameter = 2 mm) was used (Coulbourn Instruments, Holliston, MA). In every trial,
 276 the percentage of time spent “freezing” (absence of movement except respiration) was
 277 scored using FreezeFrame software (Coulbourn Instruments, Holliston, MA). The
 278 experiment took place over three days. On Day 1, mice acquired a fear conditioning
 279 response in the training context (termed context A) by receiving 5 tone-shock pairings
 280 over a 480 s. Every acquisition trial began with a 180 s habituation period wherein the
 281 mouse explored the chamber freely. At 160 s into the trial, a tone (70 dB, duration = 20
 282 s) was delivered. At 178 s into the trial, a mild footshock (0.7 mA, duration = 2 s) was
 283 delivered and co-terminated with the tone. A further four tone-signalled footshocks were
 284 delivered at 240, 300, 360, 420 s. After 480 s, the mouse was removed from the
 285 chamber and returned to their home cage for 24h. On Day 2 in the morning (9 – 12 am),
 286 contextual fear memory was measured by re-exposing mice to context A. On Day 2 in
 287 the afternoon (12 – 3 pm), contextual discrimination was measured by placing mice in a
 288 second context (context A') that was similar to context A but differed subtly in visual
 289 cues (lighting/wallpaper). Contextual discrimination was measured using the
 290 discrimination index (A' vs A), which was defined as: (Freezing in A' on Day 2) =
 291 (Freezing in A' on Day 2 + Freezing in A on Day 2). Chambers were cleaned with 70%
 292 ethanol between exposures. On day 3, cued fear conditioning was measured by re-
 293 exposing the mice to the tone in a novel chamber (context B) that differed significantly
 294 from context A in olfactory cues, visual cues and overall volume. In the cued fear
 295 conditioning trial, mice were given 180 s to explore the chamber before the tone was
 296 delivered for 300 s.

297 *Novel Object Recognition.* Mice were habituated for 15 min to the testing chamber (20 ×
 298 20 × 20 cm) before the protocol began. On the following day, each mouse was placed in
 299 the chamber for 15 min with 2 identical objects (FO1 + FO2; Training Phase). Afterward,
 300 the animal was either placed in a holding cage for 60 min (short-term version) or
 301 returned to their home cage for 24h (long-term version) (Delay Phase). During this time,
 302 the chamber was cleaned with 70% ethanol and one of the objects (FO2) was replaced
 303 with a novel object (NO). The mouse was then placed back in the chamber (Testing
 304 Phase), and the time that it spent interacting with each object was measured. Novel
 305 object preference (%) was calculated as = NO interaction / (FO1 interaction + NO

306 interaction) whereas total interaction time was calculated as = FO1 interaction + NO
 307 interaction. A within-subject design was used wherein all animals were exposed to all
 308 four conditions (vehicle/post-training, vehicle/pre-training, CNO/post-training and
 309 CNO/pre-testing) over a period of eight days in a counterbalanced order using different
 310 sets of objects. In the post-training injection condition, CNO injection was given
 311 immediately following training. In the pre-testing injection condition, CNO injection was
 312 given 15 min before the testing phase. Automated scoring was coordinated by an
 313 experimenter blind to condition using Ethovision XT 11.5 software. Animals with an
 314 interaction time of < 3 s were excluded from analysis.

315 *Puzzle box.* The box was a two-chambered apparatus consisting of a start area (58×28
 316 $\times 28$ cm³) that was brightly illuminated and a goal area ($14 \times 28 \times 28$ cm³) that was
 317 enclosed by walls and filled with bedding. Initially, the start area was connected to the
 318 goal area via a doorway and underpass. During the task, the passage to the goal area
 319 was obscured by obstacles of increasing difficulty. The task took place over 3 days with
 320 3 trials per day (D1: Trial 1 – 3, D2: Trial 4 – T6, D3: Trial 7 - T9). On the first day of
 321 testing, animals were placed in either the vehicle injection group or CNO injection group
 322 and received the same injection once per day, 10 minutes before testing began. On
 323 Trial 1 (T1), the goal area was accessible through the doorway and underpass. On T2,
 324 the doorway was blocked and the goal area was only accessible through the underpass
 325 (Underpass task). This process was repeated for T3 and T4. On T5, the doorway
 326 remained blocked and the underpass was filled with corncob bedding. To enter the goal
 327 area, the mice would have to dig through this bedding (Dig Task). This process was
 328 repeated for T6 and T7. During T8, the doorway remained blocked and the underpass
 329 was instead filled with a cardboard plug (Plug task). To enter the goal area, mice would
 330 have to remove/rearrange this plug. This process was repeated in T9. As T2, T5 and T8
 331 involved the introduction of a novel problem (Underpass, Dig task, Plug task
 332 respectively) analysis focused on these trials. In between trials, mice were placed in a
 333 holding cage while the chamber was cleaned with 70% ethanol. All scoring was done
 334 manually by an experimenter blind to condition. If a mouse failed to solve the task within
 335 300 s, the trial was terminated and a time of 300 s was assigned.

336 *Statistical analysis.* In all cases, we used a randomized experimental design. All
337 analysis was performed using GraphPad Prism 6.0 for Windows. For
338 electrophysiological experiments, the Wilcoxon matched pairs signed rank test was
339 used to compare action potential frequency before and after application of CNO, while
340 student's t-tests (paired) were performed with significance set at $p = 0.05$ for all other
341 analyses. In behavioral experiments, t-tests were generally used but two-way analysis
342 of variance (ANOVA) was employed in several cases. In open field and puzzle box
343 experiments, two-way ANOVA was used with drug as a between-subjects factor and
344 time as a within-subjects factor. In open field and elevated plus maze experiments, two-
345 way ANOVA was used with drug as a between-subjects factor and compartment as a
346 within-subjects factor. *Post-hoc* analysis for two-way ANOVA was performed using
347 Sidak's multiple comparison test with significance set at $p = 0.05$. In all experiments,
348 cases with a score of more than two absolute standard deviation units from the mean
349 were classified as outliers and excluded from analysis. This criterion resulted in the
350 exclusion of between 0 to 1 animal per experiment. All figures present data as mean \pm
351 standard error of the mean (SEM).

352

353 Results

354 Selective targeting of CCK-GABA neurons in the CCK-GABA/hM3Dq mouse line

355 To selectively target CCK-GABA neurons, we employed a dual recombinase-based
 356 (Cre and Flpe) intersectional approach (Awatramani et al., 2003; Dymecki and Kim,
 357 2007; Taniguchi et al., 2011) where neuronal subtypes are defined by overlapping
 358 expression of two genetic markers. A dual recombinase-responsive allele, *RC::FL-*
 359 *hM3Dq* (Sciolino et al., 2016), was combined with Cre and Flpe recombinase alleles,
 360 each driven by different promoters: Cre by the CCK (CCKergic) promoter and Flpe by
 361 the *Dlx5/6* (forebrain GABAergic) intergenic enhancer (Miyoshi et al., 2010). Mice that
 362 inherit all three alleles (Cre, Flpe and hM3Dq) were termed CCK-GABA/hM3Dq⁺ mice
 363 whereas mice inheriting only two alleles (Cre and hM3Dq) were termed CCK-
 364 GABA/hM3Dq⁻ mice. In CCK-GABA/hM3Dq⁺ mice, neurons expressing Cre and Flpe
 365 (i.e. CCK-GABA neurons) express the excitatory Gq-coupled receptor hM3Dq fused to
 366 the reporter protein mCherry (Figure 1A). In contrast, neurons expressing only Flpe (i.e.
 367 nonCCK-GABA neurons) express the reporter protein green fluorescent protein (GFP)
 368 but not hM3Dq receptors. CCK-GABA/hM3Dq⁻ mice do not express any reporter
 369 proteins or hM3Dq receptors (Figure 1B).

370 To demonstrate that the intersectional approach permitted selective access to
 371 CCK-GABA neurons, we quantified the expression of molecular markers consistent with
 372 CCK-GABA neuron identity in brain sections from CCK-GABA/hM3Dq⁺ mice.
 373 Histological examination confirmed an abundance of mCherry-positive cells in the
 374 *stratum radiatum* (SR), the hippocampal layer where CCK-GABA neurons are most
 375 numerous (Figure 1C) (Whissell et al., 2015). The majority of mCherry positive neurons
 376 showed immunoreactivity for CCK ($79.0 \pm 3.2\%$, $n = 11$; Figure 1D, absolute cell counts
 377 are shown in Table 1), a finding which verified the selectivity of CCK-GABA neuron
 378 targeting. Next, we evaluated the efficacy of the intersectional approach by determining
 379 the proportion of CCK-GABA neurons positive for mCherry expression. CCK-GABA
 380 neurons in CCK-GABA/hM3Dq⁺ brain sections were operationally defined as cells with
 381 immunoreactivity for the molecular markers CCK and glutamic acid decarboxylase 67
 382 (GAD67). Analysis showed that of all neurons showing immunoreactivity for CCK and

383 GAD67, the majority were also immunoreactive for mCherry ($73.6 \pm 4.6\%$, $n = 11$;
 384 Figure 1D, Table 1). To verify that the GABA neuron labelling in our mouse line was
 385 comparable to that in other intersectional models (Whissell et al., 2015) we quantified
 386 mCherry+ neurons (likely CCK-GABA neurons) and GFP+ neurons (nonCCK-GABA
 387 neurons) in the hippocampus and prefrontal cortex. Consistent with past reports, we
 388 observed that CCK-GABA neurons accounted for approximately 22% of GABA neurons
 389 in the hippocampal subregions and 19% of GABA neurons in prefrontal cortex
 390 subregions (Figure 1E, Table 1). Together, these data indicate that the CCK-
 391 GABA/hM3Dq line targets CCK-GABA neurons with relatively high selectivity and
 392 efficacy.

393 **Selective chemogenetic activation of CCK-GABA neurons in the hippocampus** 394 **increases inhibition at CA1 pyramidal neurons**

395 To verify that our model permitted selective activation of CCK-GABA neurons but not
 396 other cells, we conducted *in vitro* slice electrophysiology experiments in CCK-
 397 GABA/hM3Dq⁺ and CCK-GABA/hM3Dq⁻ mice. All experiments were performed in the
 398 SR layer of the hippocampus, where there is a relative abundance of CCK-GABA
 399 neurons available for whole-cell patch clamp (Whissell et al., 2015).

400 To determine whether CCK-GABA neurons could be reliably excited in our
 401 model, we examined the response of putative CCK-GABA neurons to clozapine-N-oxide
 402 (CNO), a compound that selectively activates transgenic hM3Dq receptors (Armbruster
 403 et al., 2007). It was expected that CNO (5 μ M) would depolarize SR cells in CCK-
 404 GABA/hM3Dq⁺ but not CCK-GABA/hM3Dq⁻ mice. In CCK-GABA/hM3Dq⁺ mice, we
 405 targeted mCherry+ neurons for whole-cell patch clamp (Figure 2A) as our histological
 406 data indicated that the majority of these cells expressed markers consistent with CCK-
 407 GABA neuron identity (Figure 1D). Consistent with expectations, CNO significantly
 408 depolarized mCherry+ neurons ($t(4) = 5.66$, $p = 0.0024$) and increased their firing
 409 frequency ($W = 45$, $p = 0.0039$). In contrast, CNO did not affect membrane potential or
 410 firing rate in mCherry- neurons from CCK-GABA/hM3Dq⁻ mice (all p values > 0.40).

411 We next verified that activation of hippocampal CCK-GABA neurons increased
 412 inhibition of their post-synaptic targets, CA1 pyramidal neurons (Basu et al., 2013). CA1

pyramidal neurons were patched and miniature inhibitory post-synaptic currents (mIPSCs) were measured before and after CNO administration (Figure 2B). In CCK-GABA/hM3Dq⁺ mice, CNO significantly increased the frequency ($t(5) = 2.82$, $p = 0.037$) but not amplitude ($p > 0.92$) of mIPSCs in CA1 pyramidal neurons. In contrast, no effects of CNO on mIPSC frequency or amplitude were observed in CA1 pyramidal neurons from CCK-GABA/hM3Dq⁻ mice (all $ps > 0.37$). Collectively, these findings indicate that CNO selectively increases the excitability of putative CCK-GABA neurons and inhibition of CA1 pyramidal neurons in CCK-GABA/hM3Dq⁺ mice.

Systemic activation of CCK-GABA neurons minimally affects anxiety but enhances memory

To determine the effects of CCK-GABA neuron activation on emotional behavior, we compared the performance of vehicle- and CNO-treated CCK-GABA/hM3Dq⁺ mice in the open field (OF), elevated plus maze (EPM) and tail suspension (TS) tests (Figure 3A-C). In the OF test, CNO treatment did not change % centre time or total distance travelled (all $ps > 0.07$, Figure 3A). Similarly, CNO treatment did not change % open arm time in the EPM (Figure 3B), which is regarded as the most reliable indicator of anxiety-like behavior (Walf and Frye, 2007). However, CNO did increase % closed arm time (two-way interaction of drug \times compartment, $F(2, 50) = 7.79$, $p = 0.0011$) a finding which may reflect a subtle increase in anxiety. To ensure that this effect of CNO was mediated by hM3Dq receptors (MacLaren et al., 2016; Gomez et al., 2017), we tested the effects of the drug in CCK-GABA/hM3Dq⁻ mice. We did not observe any effect of the drug in these animals ($F(2, 36) = 0.29$, $p = 0.75$) (Figure 3-1). In the TS test (Figure 3C), a measure of depression-like behavior, CNO had no effect on immobility time ($p > 0.67$) (Steru et al., 1985). Together, these results suggest that CCK-GABA neuron activation does not affect locomotion or depression-like behavior but mildly increases anxiety-like behaviour in CCK-GABA/hM3Dq⁺ mice.

Subtle changes in anxiety may potentially affect other behavioral functions in mice, including social behavior. Accordingly, we next examined the effects of activating CCK-GABA neurons in the three-chambered social interaction test (Moy et al., 2004). In the first portion of this test, a mouse is exposed to a novel conspecific. Interaction time

443 with this novel mouse is deemed to reflect sociability but is constrained by anxiety (File
 444 and Hyde, 1978). CNO treatment did not affect interaction time ($p > 0.86$) (Figure 3D), a
 445 finding which suggests that systemic activation of CCK-GABA cells does not affect
 446 sociability or anxiety. In the second phase of the social interaction test, we examined
 447 the effects of CNO on social recognition. To demonstrate social recognition, a mouse
 448 must exhibit a preference for a novel conspecific over a familiar conspecific (File and
 449 Hyde, 1978). Social recognition is constrained by anxiety, but also involves memory
 450 processes and is regulated by the hippocampus (Tanimizu et al., 2017), a brain region
 451 where CCK-GABA neurons are relatively abundant (Whissell et al., 2015). Surprisingly,
 452 we found that CNO enhanced social recognition in CCK-GABA/hM3Dq⁺ mice ($t(17) =$
 453 2.44 , $p = 0.026$) but not CCK-GABA/hM3Dq⁻ mice ($t(11) = 1.98$, $p = 0.078$) (Figure 3-1).
 454 These data suggest that the activation of CCK-GABA neurons enhances social
 455 recognition memory in CCK-GABA/hM3Dq⁺ mice without changing overall sociability.

456 If CCK-GABA neurons facilitated social recognition by enhancing memory
 457 processes, then activation of these cells might also improve the performance of other
 458 memory tasks. Accordingly, we determined the effects of CCK-GABA neuron activation
 459 on performance in the fear conditioning assay (Figure 3E) (Phillips and LeDoux, 1992).
 460 In this test, the animal learns to associate a stimulus (either a tone or context) with an
 461 aversive experience (an electric footshock). After multiple pairings between this stimulus
 462 and the aversive experience, the animal acquires a conditioned response (freezing) to
 463 the stimulus. This freezing response is termed conditioned fear and is indicative of
 464 learning. Given that our earlier results showed an enhancement in social recognition,
 465 we expected that conditioned fear in CCK-GABA/hM3Dq⁺ mice would be enhanced by
 466 CNO.

467 During the training session, vehicle- and CNO-treated mice showed similar levels
 468 of freezing ($p > 0.54$, Figure 3E). This finding suggested that acquisition of conditioned
 469 fear was comparable in both groups. 24h after training, mice were given a second CNO
 470 injection and re-exposed to the training context to measure contextual fear memory.
 471 Interestingly, freezing to the training context was greater in CNO-treated mice ($t(22) =$
 472 1.90 , $p = 0.036$), suggesting enhanced contextual fear memory retrieval. The next day

473 (48h after training), mice were given a third CNO injection and re-exposed to the
 474 auditory tone to measure cued fear conditioning. This behavior was not affected by
 475 CNO ($t(11) = 0.39$, $p = 0.035$). These results indicated that CCK-GABA neuron
 476 activation enhances the retrieval of contextual fear memory.

477 To exclude the possibility that anxiety influenced the contextual fear memory in
 478 CCK-GABA/hM3Dq⁺ mice, we next measured contextual discrimination (Whissell et al.,
 479 2013). In this task, mice are exposed to a novel context (A') that is highly similar to the
 480 training context (A) in features. Mice with strong contextual memories show selectively
 481 increased freezing in the training context but not the similar context. In contrast, anxious
 482 mice typically show high freezing in both contexts due to fear-induced stimulus
 483 generalization (Huckleberry et al., 2016). Memory performance in this task is evaluated
 484 using the discrimination ratio ($d = \text{freezing in A'} / [\text{freezing in A} + \text{freezing A'}]$, ranging
 485 from 0 to 1). Contextual discrimination is notoriously difficult; mice may take 9 days or
 486 more to learn this behavior (Whissell et al., 2013). Surprisingly, CNO-treated mice
 487 exhibited successful context discrimination after one day of training ($t(22) = 1.98$, $p =$
 488 0.030 , Figure 3E). These results support the argument that enhanced fear conditioning
 489 with CCK-GABA neuron activation is due to enhanced memory rather than increased
 490 anxiety.

491 **Activation of CCK-GABA neurons enhances novel object recognition**

492 The above results suggested that CCK-GABA neuron activation is associated with
 493 improved memory performance in several tasks. However, it was unclear how CCK-
 494 GABA neuron activation bolsters memory performance. Memory processing is argued
 495 to be composed of several stages – acquisition, maintenance/consolidation and retrieval
 496 – each of which may be modulated by CCK-GABA neuron activity. To determine
 497 whether memory maintenance/consolidation or retrieval are modulated by CCK-GABA
 498 activity, we examined how CNO injection during these stages affected memory in the
 499 novel object recognition task (Figure 4A) (Warburton and Brown, 2015).

500 In this task, the mouse is placed in an arena where it is allowed to interact with
 501 two identical objects (training; where object memories are acquired). In the second
 502 phase, the mouse is removed from the arena for 1 hr (post-training; where memories

are maintained/consolidated). In the third phase, the mouse is returned to the arena, which now includes a familiar object from the training phase and a novel object (testing; where object memories are retrieved). Mice that have a strong memory of the familiar object show a preference for the novel object and interact with it more frequently. To discern the effects of CNO on different phases of memory processing, CCK-GABA/hM3Dq⁺ mice were given drug injections in the post-training or pre-testing period. We expected that the pre-testing injection of CNO would enhance object preference in the novel object recognition test, as pre-testing injection of CNO enhanced the retrieval of contextual fear memory in the fear conditioning task.

Post-training injections did not affect novel object preference ($p > 0.34$), but tended to reduce object interaction ($p = 0.053$) (Figure 4B). The failure of post-training CNO to enhance object recognition does not support the notion that CCK-GABA neuron activation affects memory maintenance/consolidation. The pre-testing injection was given 15 min prior to testing, as CNO takes ~15 min to reach peak levels in plasma (Guettier et al., 2009). In contrast, pre-testing CNO injection significantly increased novel object preference ($t(11) = 1.78$, $p = 0.05$) but did not affect object interaction ($p > 0.22$) (Figure 4C). The memory-enhancing effects of pre-testing CNO required the expression of hM3Dq receptors, as CCK-GABA/hM3Dq⁻ mice were unaffected by CNO ($t(12) = 0.75$, $p = 0.23$) (Figure 3-1). Collectively, these results suggested that CCK-GABA neuron activation enhances the retrieval of memories acquired one hour earlier. To verify that CCK-GABA neuron activation also enhanced the retrieval of long-term memories, we investigated the effect of CNO on object recognition measured 24h after training. Consistent with our previous result, CNO enhanced object recognition measured 24h after training in CCK-GABA/hM3Dq⁺ mice ($t(11) = 2.13$, $p = 0.028$) but did not affect interaction time ($p = 0.34$) (Figure 4D).

Activation of CCK-GABA neurons enhances performance in the puzzle box test

The above results showed that the activation of CCK-GABA neurons enhanced performance in three different memory assays: social recognition, contextual fear conditioning and novel object recognition (Figure 3D, E; Figure 4). However, an

532 interesting question is whether CCK-GABA neuron activation enhances performance in
533 a cognitive task that does not directly depend upon memory processes.

534 To determine the effects of CCK-GABA neuron activation on cognitive
535 performance, we subjected CCK-GABA/hM3Dq⁺ mice to the puzzle box test. This assay
536 is regarded as an animal model of executive function, cognitive flexibility and problem-
537 solving. The test takes place in a two-chambered apparatus: one chamber is brightly
538 illuminated and exposed to air ('start area') whereas the other chamber is enclosed by
539 walls and filled with bedding ('goal area') (Figure 5A). The mouse begins in the start
540 area and must navigate to the goal area to complete the task. However, over a series of
541 trials, the path to the goal area is blocked by a series of increasingly difficult obstacles.
542 To reach the goal area, mice must develop strategies to overcome these obstacles. In
543 trial 2, mice must learn to use an underpass (Underpass Task). In trial 5, the underpass
544 is blocked with bedding and mice must dig through this bedding (Dig Task). In trial 8,
545 the underpass is blocked with a plug and mice must remove this plug (Plug Task). As
546 the obstacle in each of these trials (T2, T5 and t8) is novel, a mouse cannot rely on a
547 previously learned strategy (or memory) and must develop a new approach to the
548 problem. If CCK-GABA neuron activation enhanced cognitive function, it is likely that
549 CNO-treated mice would complete trials more quickly than vehicle-treated mice. Indeed,
550 CNO-treated mice generally performed all three tasks more quickly than vehicle-treated
551 mice (main effect of drug, $F(1, 23) = 4.70$, $p = 0.041$) (Figure 5B). However, *post-hoc*
552 analysis indicated that CNO-treated mice only performed significantly better than
553 vehicle-treated controls on the Plug Task (T8) (Figure 5B). This enhanced performance
554 was not due to CNO specifically, as CCK-GABA/hM3Dq⁻ mice did not respond to the
555 drug ($F(1, 9) = 2.74$, $p = 0.13$) (Figure 3-1). These data support the notion that CCK-
556 GABA neuron activation facilitates general cognitive function as well as memory
557 performance.

558

559 Discussion

560 In the present study, we used a dual-recombinase strategy to selectively activate CCK-
561 GABA neurons during an array of emotional and cognitive behaviors. Histological and
562 electrophysiological data showed highly effective targeting and CNO-mediated
563 activation of CCK-GABA cells, respectively. Behavioural data revealed that the
564 activation of CCK-GABA neurons enhances memory and cognitive performance in
565 CCK-GABA/hM3Dq⁺ mice with minimal effects on anxiety. As a recent study showed
566 that CNO is converted to clozapine and clozapine can affect the brain without binding to
567 hM3Dq receptors (Gomez et al., 2017), we also investigated whether CNO had effects
568 that were not dependent upon hM3Dq receptors. As we did not observe any effects of
569 CNO in CCK-GABA/hM3Dq⁻ mice, it is most likely that our behavioral effects are
570 mediated by the activation of hM3Dq receptors expressed in CCK-GABA cells.

571 To our knowledge, this is the first report which directly reveals a selective
572 contribution of CCK-GABA neuron activity to enhancement of cognitive performance.
573 How might CCK-GABA neuron activation facilitate cognition and memory? The signaling
574 by CCK-GABA neurons is complex as it involves both the inhibitory neurotransmitter
575 GABA and the neuropeptide CCK, which tends to be excitatory (Lee et al., 2011). In the
576 hippocampus, the release of GABA from CCK-GABA neurons inhibits CA1 pyramidal
577 cells (Basu et al., 2013) whereas the release of CCK excites parvalbumin-expressing
578 GABA neurons (Lee et al., 2011). As parvalbumin-expressing GABA neurons also
579 inhibit CA1 pyramidal cells, both these effects may ultimately increase inhibitory tone
580 within the hippocampus. Several studies have demonstrated that inhibitory tone
581 supports hippocampus-dependent memory processes, including contextual
582 discrimination (Whissell et al., 2013; Engin et al., 2015), a behavior which was
583 increased by CCK-GABA neuron activation in the present study. Contextual
584 discrimination is thought to require the orthogonal representation of contextual
585 memories in non-overlapping populations of neurons (Aimone et al., 2011). By
586 increasing inhibition, CCK-GABA neurons may effectively reduce 'noise' in the
587 hippocampus, making it possible to encode orthogonal memories that are easily
588 discriminated. Through their inhibitory actions in the hippocampus, CCK-GABA neurons

589 may play a role in ‘signal amplification’ by enhancing the signal-to-noise ratio at the
590 network level (Bartos and Elgueta, 2012).

591 Additionally, CCK-GABA neurons may contribute to memory processes via
592 effects on other forms of network processing. Most notably, CCK-GABA neurons may
593 contribute to the generation of theta oscillations, which are linked to cognition and
594 memory (Curley and Lewis, 2012). Inhibition of a GABAergic cell population which
595 includes CCK-GABA neurons impairs theta oscillations in the cortex and hippocampus
596 (Nagode et al., 2014; Nguyen et al., 2014), an effect associated with impaired memory
597 (Raver and Keller, 2014). Accordingly, several of the behavioral effects shown here may
598 be explained by CCK-GABA neuron-mediated sculpting of cortical oscillations. As we
599 did not examine cortical oscillations here, this exciting possibility may be addressed in
600 future studies.

601 Our model, which permits CCK-GABA neuron activation via hM3Dq receptors,
602 does not allow for the specific control of CCK or GABA release from these cells.
603 However, it is most likely that CCK and GABA neurotransmission jointly contribute to
604 our behavioral effects. Most notably, the cluster of behaviors affected in this study
605 differs considerably from those previously linked to GABAergic transmission in CCK-
606 GABA neurons (Brown et al., 2014; Schmidt et al., 2014). In one transgenic model,
607 GABA synthesis was inhibited in CB1 receptor-expressing cells (Brown et al., 2014) the
608 majority of which are CCK-GABA neurons (Marsicano and Lutz, 1999). In contrast to
609 our findings, this manipulation enhanced cued fear but did not affect contextual fear
610 learning (Brown et al., 2014). In another report, inhibition of GABA synthesis in CCK-
611 expressing cells disrupted olfaction and locomotion but not recognition memory or
612 anxiety (Schmidt et al., 2014). While these models and our own are different in the
613 extent and direction to which they manipulate GABA release (Brown et al., 2014;
614 Schmidt et al., 2014), they illustrate that GABA release accommodates only part of
615 CCK-GABA neuron functionality. Notably, CCK release from CCK-GABA neurons may
616 be linked to the subtle increase in anxiety-like behaviours observed in our study and
617 other models (Bowers and Ressler, 2015). As CCK and GABA signaling interact *in vivo*
618 (Lee et al., 2011) it may be impractical to separate them experimentally.

619 An important consideration is that the CCK-GABA/hM3Dq model used here
620 permits only global activation of CCK-GABA neurons. As CNO was injected in the
621 intraperitoneal cavity and processed systemically, it activates CCK-GABA neurons
622 across the entire forebrain. Though multiple CCK-GABA neuron populations may
623 contribute to the behaviors studied here, we propose that CCK-GABA neurons in the
624 hippocampus play an important role. Importantly, our electrophysiological data shows
625 that CCK-GABA neuron activation is associated with increased inhibition of CA1
626 pyramidal neurons in the hippocampus. Many behaviors enhanced by CCK-GABA
627 neuron activation (including contextual discrimination, contextual fear conditioning and
628 puzzle box performance) are regulated by the hippocampus (Phillips and LeDoux, 1992;
629 Frankland et al., 1998; Ben Abdallah et al., 2011). CNO-mediated enhancement of
630 performance in these tasks may arise from altered hippocampal network behaviour, as
631 a recent study found that disruption of CCK-GABA neuron wiring in the hippocampus
632 was associated with abnormal neuronal oscillations, dysregulated spatial memory
633 encoding and impaired learning (Del Pino et al., 2017). Similarly, others have shown
634 that indirect inhibition of CCK-GABA neurons in the CA1 region altered contextual fear
635 conditioning and object recognition (Basu et al., 2016). Though hippocampal CCK-
636 GABA neurons are likely involved in our behavioral effects, it is also possible that other
637 CCK-GABA populations contribute. CCK-GABA neurons in the medial prefrontal and
638 perirhinal cortex (Whissell et al., 2015) may be particularly relevant to the enhanced
639 recognition memory observed in CNO-treated CCK-GABA/hM3Dq mice, as recognition
640 memory depends upon these areas (Warburton and Brown, 2015). Ultimately, functional
641 characterization of individual populations of CCK-GABA neurons will require a
642 comprehensive, region-specific approach. In this regard, selective delivery of CNO to
643 individual brain regions through cannulation may be productive.

644 Several disorders may involve disruption of CCK-GABA neuron signaling,
645 including chronic stress (Reich et al., 2013) and schizophrenia (Curley and Lewis,
646 2012). In the case of schizophrenia, evidence suggests that dysregulated CCK-GABA
647 neuron signaling may contribute to the pathogenesis of the disorder (Curley and Lewis,
648 2012; Nguyen et al., 2014). In human studies of schizophrenia and animal models of the
649 disorder, there is reduced expression of CCK and CB1 receptor markers (Eggan et al.,

650 2008; Hashimoto et al., 2008) as well as suppression of the cortical rhythms mediated
651 by CCK-GABA neurons (Raver and Keller, 2014). Our data support a role for the
652 dysregulation of CCK-GABA activity in cognitive dysfunction associated with these
653 disorders. If disrupted CCK-GABA neuron signaling contributes to the pathogenesis of
654 schizophrenia, then activation of CCK-GABA neurons might prove therapeutic. The
655 CCK-GABA/hM3Dq model provides a useful strategy for testing this possibility.

656

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- 810

811 **TABLE LEGEND**

812 Table 1. Absolute cell counts of mCherry- and GFP-labelled cells in the hippocampus
813 and prefrontal cortex of hM3Dq+ mice. mCherry-labelled cells represent probable CCK-
814 GABA neurons whereas GFP-labelled cells represent nonCCK-GABA neurons.

815

816 **FIGURE LEGENDS**

817 **FIGURE 1.** Intersectional genetic approach for targeting hM3Dq receptors to CCK-
818 GABA neurons. *A, Top.* The dual recombinase-responsive *RC::FL-hM3Dq* allele is
819 knocked-in to the Gt(ROSA)26Sor (R26) locus with CAG (chicken β -actin and CMV
820 enhancer) promoter elements. *A, Middle.* Flpe-mediated excision of the FRT
821 (rectangles, denoted by *F*)-flanked stop cassette (STOP) permits expression of GFP.
822 Transcription of the mCherry-hM3Dq allele is prevented by the inverted orientation of
823 the sequence and second stop cassette. *A, Bottom.* Subsequent Cre-mediated
824 recombination of loxP (red triangles) and lox2272 (blue triangles) sites constituting the
825 Cre- dependent FLEX switch leads to the removal of the GFP sequences and second
826 stop cassette as well as inversion of the hM3Dq-mCherry sequences into the proper
827 orientation for transcription. Cells expressing Cre and Flpe therefore express hM3Dq-
828 mCherry but not GFP. *B.* Venn diagrams illustrating intersectional and subtractive cell
829 populations targeted using the intersectional genetic approach. The *Dlx5/6-Flpe* allele is
830 specific to GABAergic cells in the forebrain whereas the *CCK-Cre* allele is specific to
831 Cre-expressing cells. In mice inheriting all three alleles (*CCK-Cre;Dlx5/6-Flpe;RC::FL-*
832 *hM3Dq*), cells expressing both Cre and Flpe alleles (i.e. intersectional population)
833 represent CCK-GABA neurons and express hM3Dq-mCherry. Cells expressing only the
834 Flpe allele (i.e. subtractive population) represent nonCCK-GABA neurons and express
835 only GFP. *C.* Low magnification images showing hM3Dq-mCherry+ and GFP+ neurons
836 in the dorsal hippocampus of CCK-GABA/hM3Dq+ mice. Scale bar = 250 μ m. *D.*
837 Confocal images of the CA1 *stratum radiatum* in CCK-GABA/hM3Dq+ mice. mCherry+
838 cells are labelled in red, CCK+ cells are labelled in blue and GAD67+ cells are labelled
839 in green. Cells expressing all three markers (mCherry, CCK and GAD67) represent

likely CCK-GABA neurons. Scale bar = 25 μ m. *E.* Quantification of hM3Dq-mCherry+ and GFP+ cells in the hippocampus (n = 4) and prefrontal cortex (n = 5) of CCK-GABA/hM3Dq⁺ mice. Shown is the percentage of hM3Dq-mCherry+ cells out of all labelled cells. Scale bar = 500 μ m. *Abbreviations:* dCA1 = dorsal CA1, vCA1 = ventral CA1, dCA3 = dorsal CA3, vCA3 = ventral CA3, dDG = dentate gyrus, Cg = Cingulate, DP = Dorsal Peduncular Cortex, IL = Infralimbic Cortex and PL = Prelimbic Cortex. All figures present data as mean \pm SEM.

FIGURE 2. Selective activation of CCK-GABA neurons increases inhibition of CA1 pyramidal neurons in CCK-GABA/hM3Dq mice. *A.* Whole cell patch-clamp recordings of CA1 *stratum radiatum* (SR) neurons in CCK-GABA/hM3Dq⁻ (*top*) and CCK-GABA/hM3Dq⁺ mice (*bottom*) with and without CNO. In SR neurons from CCK-GABA/hM3Dq⁺ mice only, the administration of CNO (5 μ M) significantly depolarized the membrane potential (n = 5) and increased firing rate (n = 13). *B.* Whole cell patch-clamp recordings from CA1 pyramidal cells (PCs) in CCK-GABA/hM3Dq⁻ (*top*) and CCK-GABA/hM3Dq⁺ mice (*bottom*) with and without CNO. In CA1 PC neurons from CCK-GABA/hM3Dq⁺ only (n = 6), CNO increased the frequency but not amplitude of mIPSCs. *Abbreviations:* aCSF = artificial cerebrospinal fluid, mIPSC = miniature inhibitory post-synaptic current. All figures present data as mean \pm SEM.

FIGURE 3. CCK-GABA neuron activation minimally affects overall emotional behavior but enhances contextual fear memory. *A.* Open field test. CNO-treated mice (*red*, n = 10) do not differ from vehicle-treated mice (*black*, n = 9) in center time, periphery time or distance traveled. *B.* Elevated Plus Maze. CNO-treated mice (n = 13) show significantly higher closed arm time than vehicle-treated controls (n = 14). However, open and

863 closed arm time was not significantly different between groups. *C.* Tail suspension test.
864 Immobility time, an indicator of behavioral despair and depression-like behavior, does
865 not differ between vehicle-treated ($n = 6$) and CNO-treated animals ($n = 7$). *D.* Social
866 interaction test. Preference for novel mice, an indicator of social recognition, is higher in
867 CNO-treated animals ($n = 10$) than vehicle controls ($n = 10$). Interaction time is
868 unaffected by CNO. *E.* Fear conditioning and contextual discrimination. *Top, Left.*
869 Experimental protocol. *Top, right.* CNO- ($n = 13$) and vehicle-treated ($n = 11$) animals
870 show similar responses during training sessions. *Bottom, Left.* Recall of contextual but
871 not cued fear conditioning, as evidenced by percentage freezing, is significantly greater
872 in CNO-treated mice. *Bottom, Right.* CNO-treated mice show greater contextual
873 discrimination than vehicle-treated mice. All figures present data as mean \pm SEM.
874 Behavioral data in hM3Dq- mice is given in Extended Data, Figure 3-1.

875 FIGURE 4. Selective activation of CCK-GABA neurons enhances novel object
876 recognition memory. *A.* Experimental protocol showing training (15 min), delay (1 h or
877 24 h) and testing (15 min) phases. Post-training injections were given immediately
878 following training whereas pre-testing injections were given 15 min prior to testing. *B.*
879 Post-training CNO injection did not affect novel object preference ($n = 11$) but tended to
880 reduce interaction time. *C.* In the 1 h protocol, pre-testing CNO injection increased novel
881 object preference ($n = 12$) but did not affect interaction time. *D.* In the 24 h protocol, pre-
882 testing CNO injection increased novel object preference ($n = 12$) but not affect
883 interaction time. All figures present data as mean \pm SEM. Behavioral data in hM3Dq-
884 mice is given in Extended Data, Figure 3-1.

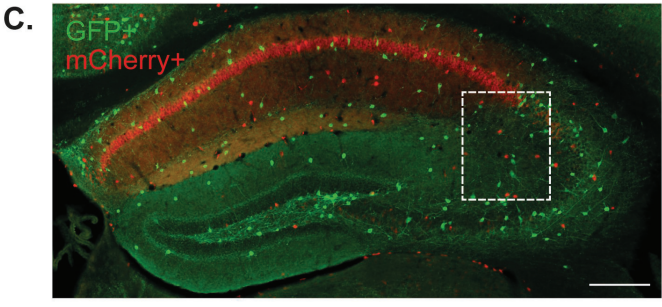
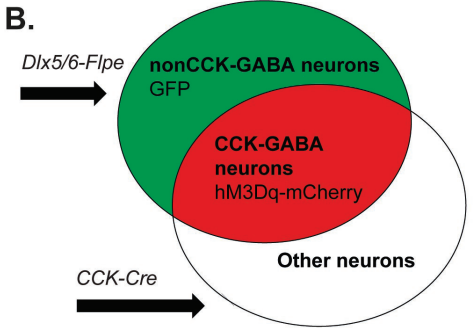
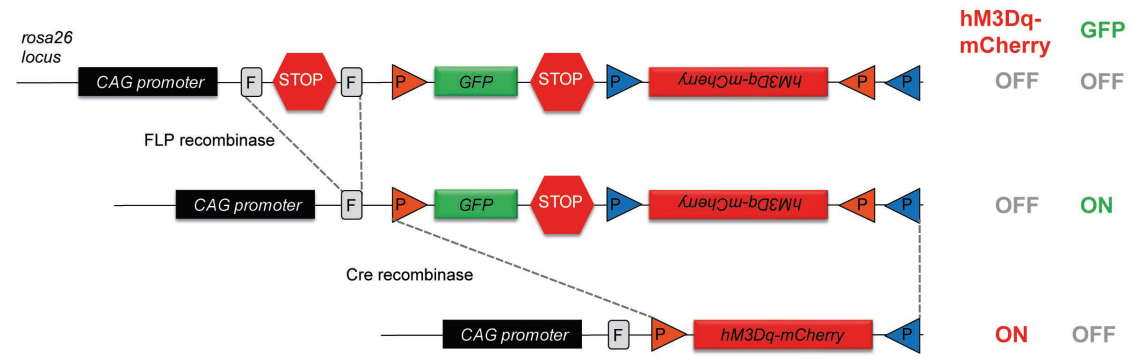
885 FIGURE 5. Selective activation of CCK-GABA neurons enhances performance in the
886 puzzle box test. *A.* Cartoon of the puzzle box test showing all trials in which a new
887 obstacle is introduced (Trial 2: Underpass; Trial 5: Dig; Trial 8: Plug). In these trials, the
888 mouse must acquire a new strategy to escape from the start area into the goal area. *B,*
889 *Left.* Escape latency of CNO- ($n = 11$) and vehicle-treated ($n = 14$) CCK-GABA/hM3Dq
890 mice in all nine trials of the puzzle box test. Lower escape latencies indicate better
891 performance. CNO-treated mice show significantly lower escape latency only in Trial 8
892 (Plug task). *B, Right.* Escape latencies for CNO- and Veh-treated mice in all novel
893 obstacle trials (Underpass, Dig and Plug task). All figures present data as mean \pm SEM.
894 Behavioral data in hM3Dq- mice is given in Extended Data, Figure 3-1.

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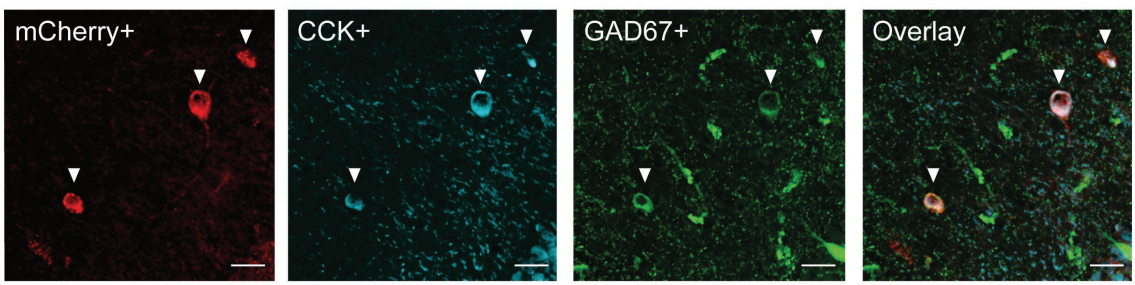
896 Extended Data, Figure 3-1. Lack of behavioral response to CNO in hM3Dq- mice. *A.*
897 Elevated Plus Maze. CNO-treated mice ($n = 10$) do not differ from Veh-treated controls
898 ($n = 10$) in performance. *B.* Social interaction test. Preference for novel mice does not
899 differ between CNO-treated animals ($n = 6$) and vehicle controls ($n = 7$). *C.* Novel object
900 recognition test. CNO-treated animals ($n = 13$) and Veh-treated animals ($n = 13$) did not
901 differ in object recognition (*left*) or interaction time (*right*). *D.* Puzzle box. Escape
902 latencies for CNO- ($n = 5$) and Veh-treated mice ($n = 6$) is comparable in all trials. All
903 figures present data as mean \pm SEM.

904

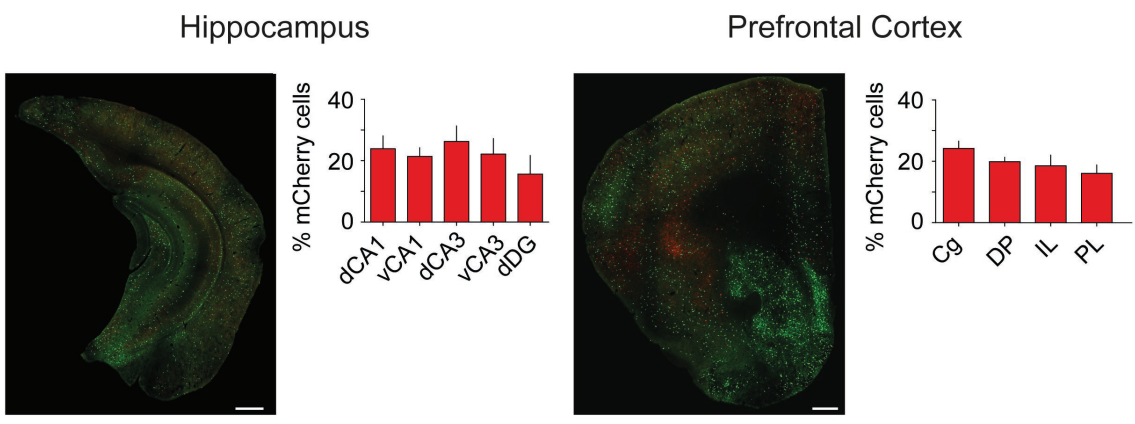
A. Intersectional Genetic Targeting of CCK-GABA neurons



D. Selectivity and Efficacy of CCK-GABA neuron targeting

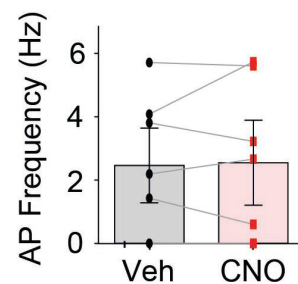
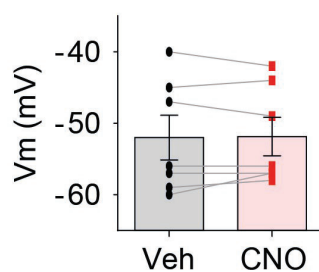
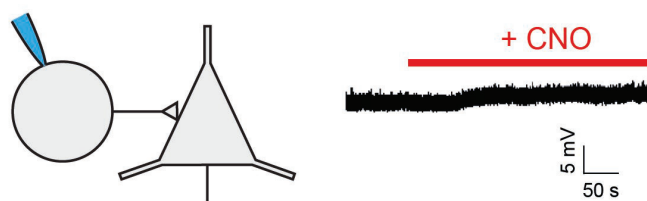


E. Quantification of CCK-GABA neuron labeling

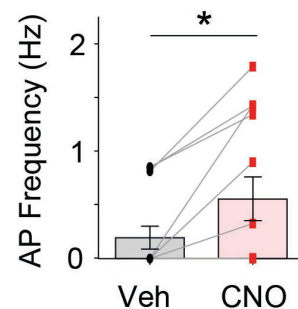
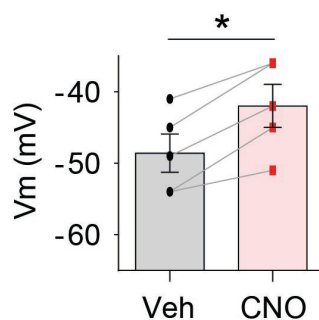
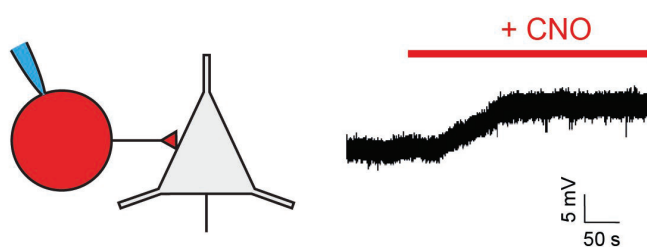


A

hM3Dq- CA1 SR Neuron

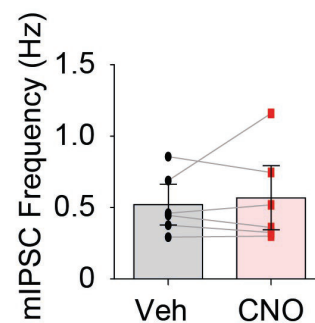
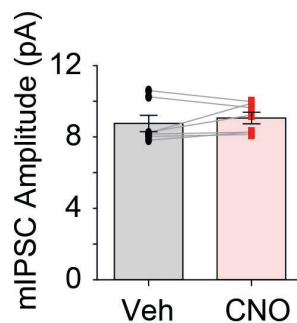
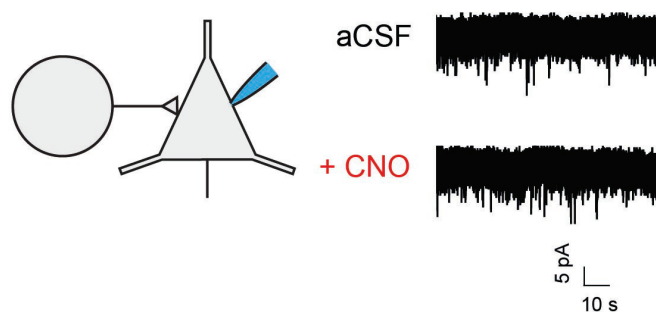


hM3Dq+ CA1 SR Neuron

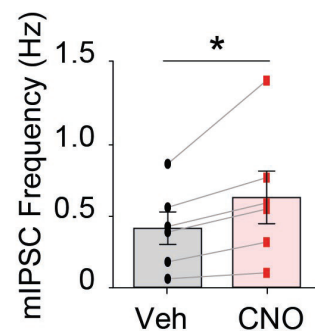
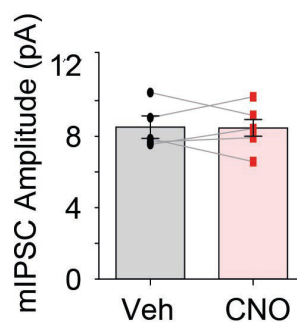
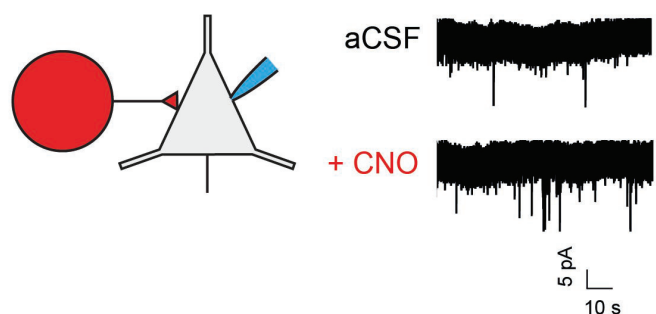


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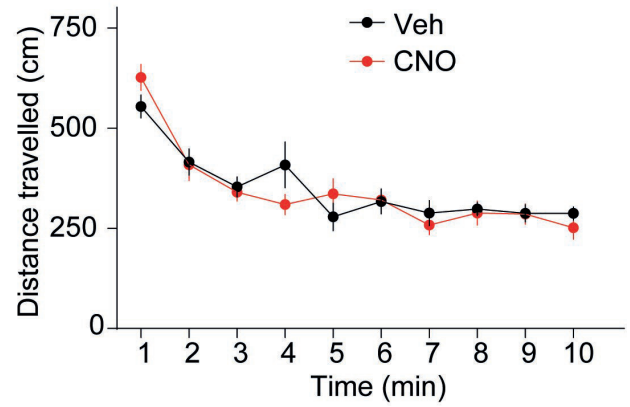
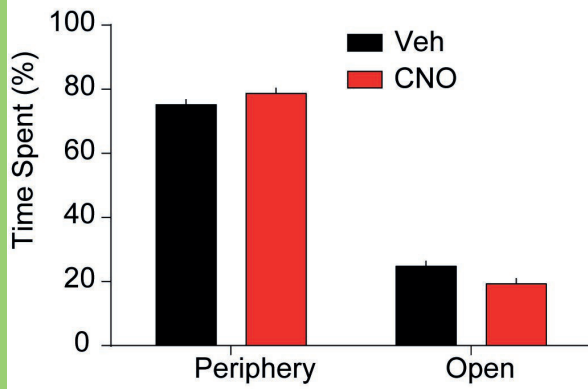
hM3Dq- CA1 PC Neuron



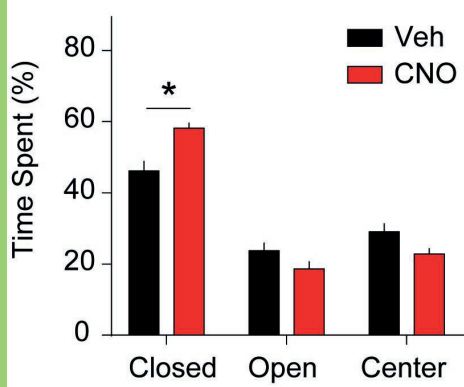
hM3Dq+ CA1 PC Neuron



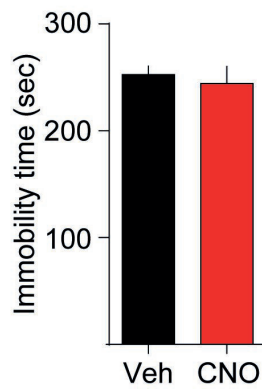
A. Open Field



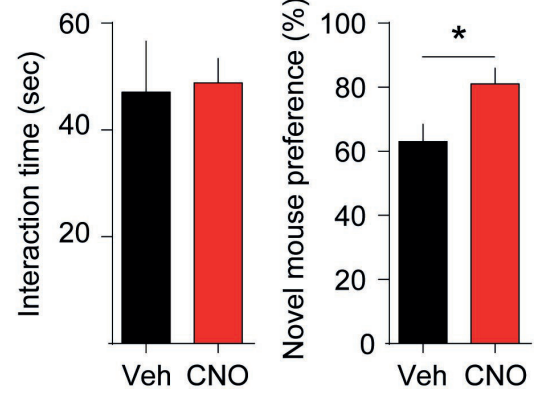
B. Elevated Plus Maze



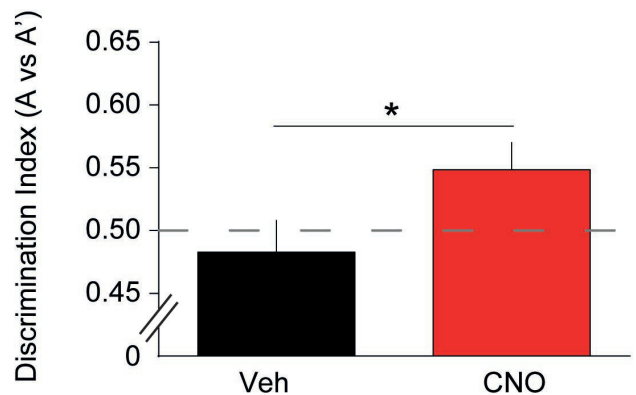
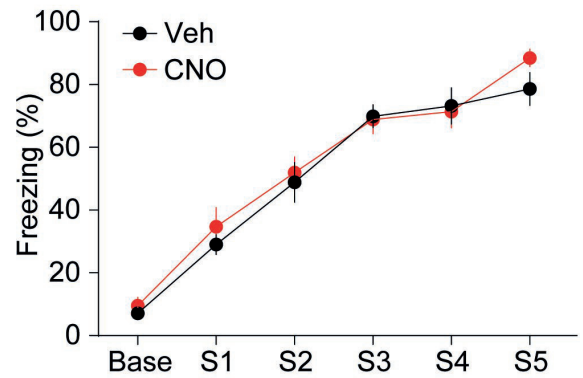
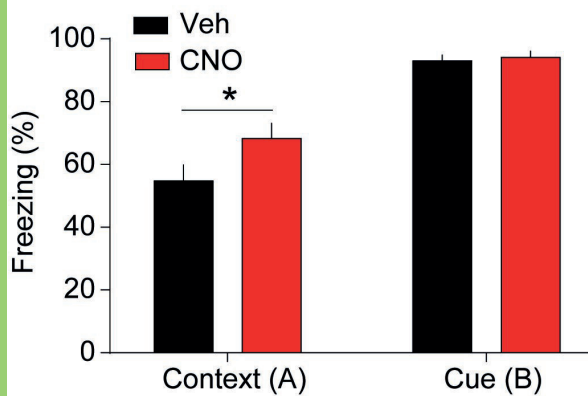
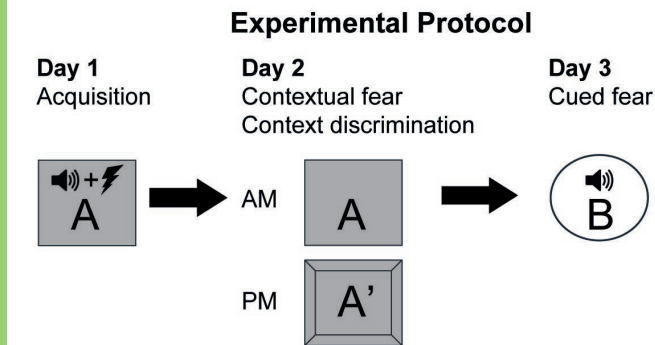
C. TST



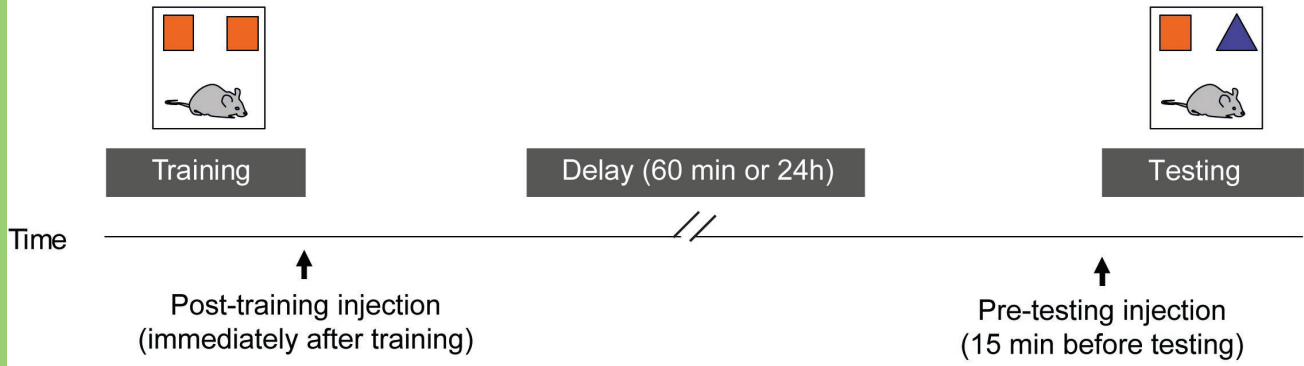
D. Social Interaction Test



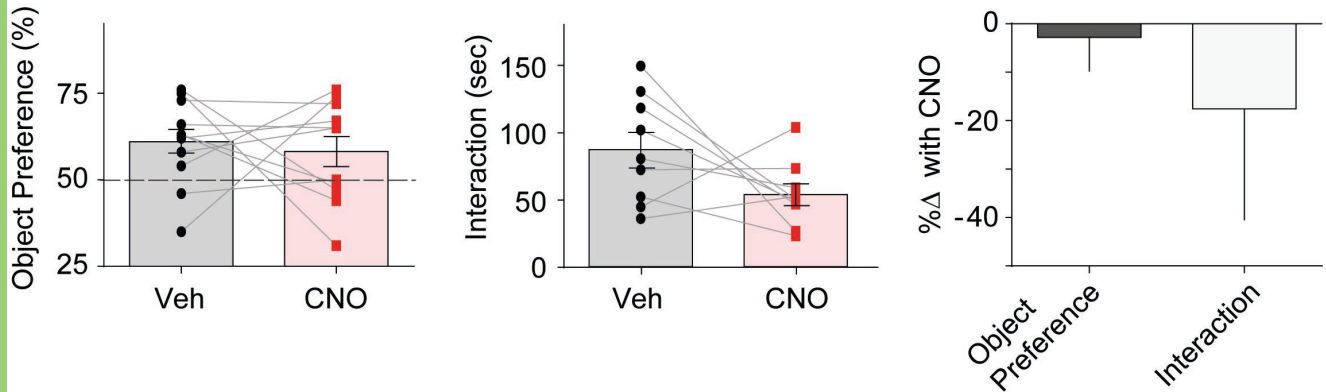
E. Fear Conditioning



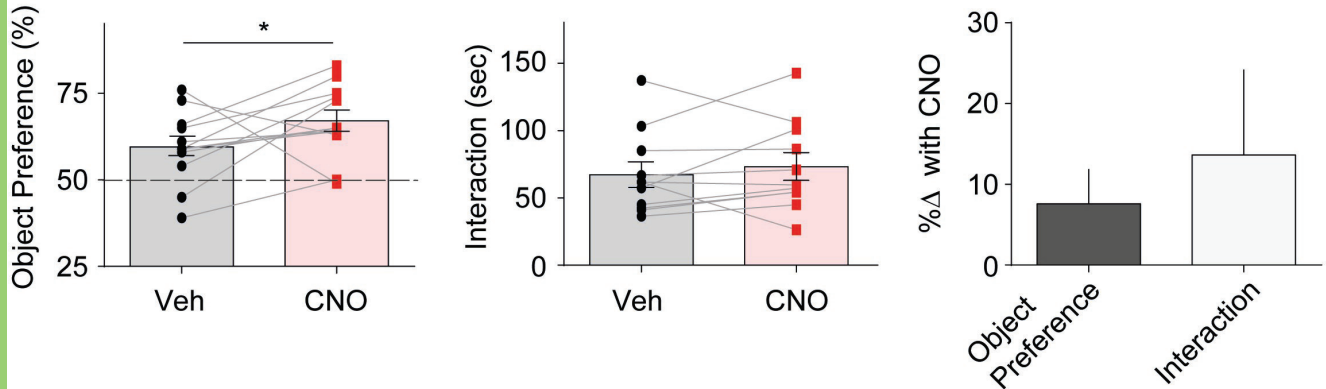
A. Experimental Protocol



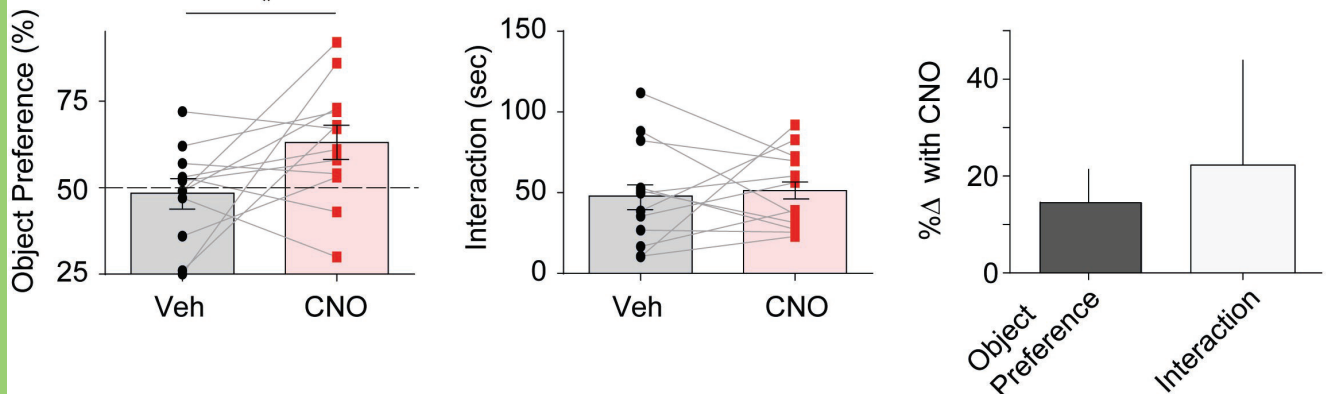
B. Post-training injection



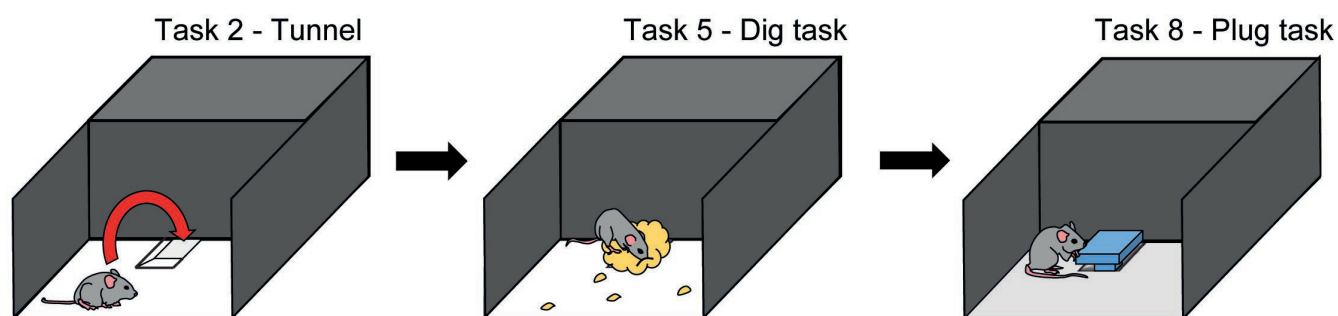
C. Pre-testing injection (60 min delay)



D. Pre-testing injection (24h delay)



A. Experimental Paradigm



B. Trial Latency

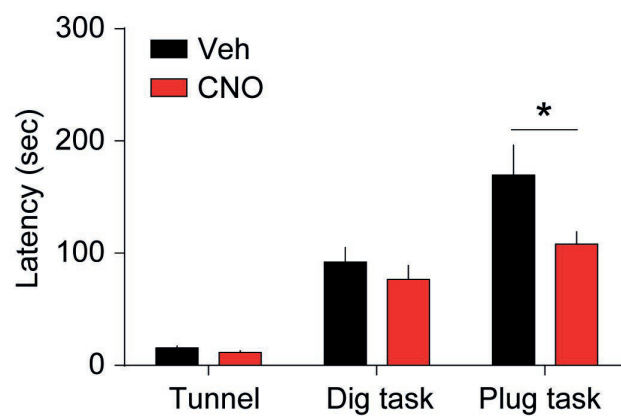
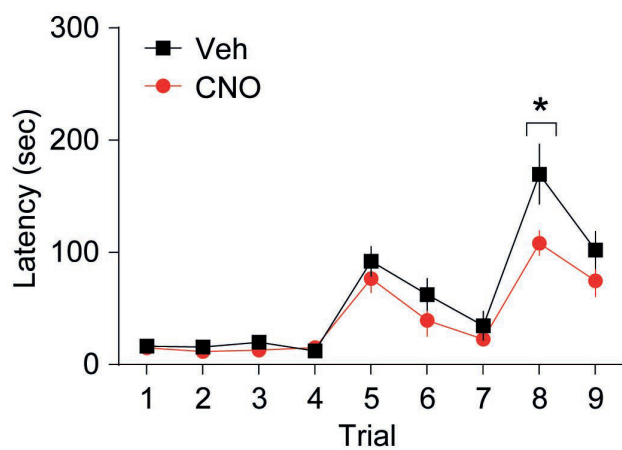


Table 1. Absolute cell counts of mCherry- and GFP-labelled cells in the hippocampus and prefrontal cortex of hM3Dq+ mice.

Main region	Subregion		mCherry-labelled cells		GFP-labelled cells	
			Absolute count		Absolute count	
			Mean	SEM	Mean	SEM
Hippocampus	dCA1	CA1, dorsal	31.4	8.8	94.8	15.5
	vCA1	CA1, ventral	22.9	3.2	87.0	19.5
	dCA3	CA3, dorsal	16.9	2.1	48.7	8.4
	vCA3	CA3, ventral	15.9	1.8	56.2	4.2
	dDG	Dentate gyrus, dorsal	15.1	6.7	73.7	17.0
Frontal cortex	Cg	Cingulate	18.6	0.4	57.6	0.4
	DP	Dorsal penducular	8.4	0.1	37.8	0.6
	IL	Infralimbic	10.8	0.4	56.6	0.9
	PL	Prelimbic	23.8	0.3	103.3	1.4