Psychopharmacology
https://doi.org/10.1007/s00213-020-05560-6

ORIGINAL INVESTIGATION

Cocaine reward and memory after chemogenetic inhibition of distinct serotonin neuron subtypes in mice

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Received: 14 January 2020 / Accepted: 18 May 2020

Abstract

Rationale We probed serotonin neurons, those denoted by their developmental gene expression as *r2Hoxa2-Pet1* (experiment 1) and *Drd1a-Pet1* (experiment 2), for differential modulation of cocaine reward and memory as revealed by the expression and development of conditioned place preference (CPP) in transgenic mice.

Objectives To query roles in CPP, we inhibited neurons cell autonomously in vivo by activating the transgenically expressed, synthetic DREADD receptor hM_4Di (Di) with the exogenous ligand clozapine-N-oxide (CNO).

Methods To examine CPP expression, mice were conditioned using behaviorally active doses of cocaine (10.0 or 17.8 mg/kg) vs. saline followed by CPP assessment, first without neuron inhibition (post-conditioning session 1), and then with CNO-mediated neuron inhibition (post-conditioning session 2), followed by 4 more post-conditioning sessions. To examine CPP development, we administered CNO during conditioning sessions and then assayed CPP across 6 post-conditioning sessions.

Results In *r2Hoxa2-Pet1-Di* mice, post-conditioning CNO administration did not impact cocaine CPP expression, but after CNO administration during conditioning, cocaine CPP (17.8 mg/kg) persisted across post-conditioning sessions compared with that in controls, suggesting a deficit in extinguishing cocaine memory. *Drd1a-Pet1-Di* mice, prior to CNO-Di-triggered neuronal inhibition, unexpectedly expressed heightened cocaine CPP (10.0 and 17.8 mg/kg) compared with controls, and this basal phenotype was transiently blocked by acute post-conditioning CNO administration and persistently blocked by repeated CNO administration during conditioning.

Conclusion Cocaine reward and memory likely map to distinct serotonergic *Pet1* neuron subtypes. *r2Hoxa2-Pet1* neurons normally may limit the durability of cocaine memory, without impacting initial cocaine reward magnitude. *Drd1a-Pet1* neurons normally may help to promote cocaine reward.

Keywords Clozapine-N-oxide · Cocaine · Conditioned place preference · *Drd1a-Pet1* neurons · Inhibitory DREADD · Locomotor activity · *r2Hoxa2-Pet1* neurons

Introduction

In a recent national survey, nearly 1 million Americans met diagnostic criteria for cocaine dependence (Center for Behavioral Health Statistics and Quality, 2015). Mechanistically, cocaine blocks dopamine, norepinephrine, and serotonin (5-hydroxytryptamine; 5-HT) reuptake at monoamine membrane transporters (Fleckenstein et al., 2000). Much research has focused on dopamine (Volkow et al. 2007), but recently, efforts have been directed toward the role of 5-HT as a means to develop new therapeutic approaches for cocaine dependence (Devroye et al. 2013; Howell and Cunningham, 2015). Using intravenous cocaine self-administration, which measures drug reinforcement and

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00213-020-05560-6) contains supplementary material, which is available to authorized users.

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abuse liability (Panlilio and Goldberg, 2007), studies demonstrated reductions in self-administration and reinstatement after systemic or focal brain injections of 5-HT_{2C} receptor agonists and 5-HT_{2A} receptor antagonists (Fletcher et al., 2004; Burbassi and Cervo, 2008; Pentkowski et al., 2010; Cunningham et al. 2011; Fletcher et al. 2011; Pockros et al. 2011; Cunningham et al. 2013). Correspondingly, development of cocaine-conditioned place preference (CPP), which measures drug reward and memory (Bardo and Bevins, 2000), is reduced after systemic injection of a 5-HT_{2C} receptor agonist (Craige and Unterwald, 2013). These are promising leads for medication development, although relatively global brain modulation due to receptor distribution may be unnecessary or undesirable in the treatment of cocaine dependence.

Advances in dissecting physiological and behavioral functions of distinct sets of 5-HT neurons have been made, with multiple molecular subtypes of 5-HT neurons identified by gene expression (Wylie, et al., 2010; Jensen et al., 2008; Okaty et al. 2015; Spaethling et al., 2014) and characterized by innervation profiles and in some cases even function at the organismal level (Brust et al., 2014; Muzerelle et al., 2016; Teissier et al., 2015; Niederkofler et al. 2016; Hennessy et al. 2017). We selected two 5-HT neuronal subtypes to study cocaine reward and memory with innervation profiles suggestive of specialized roles in behavior modulation. One subtype, referred to as *r2Hoxa2-Pet1* neurons, originates from rhombomere 2 of the embryonic hindbrain and expresses transcription factor-encoding genes *Hoxa2* and *Pet1* through adulthood (Jensen et al., 2008; Okaty et al., 2015). HOXA2 is an evolutionarily conserved fate-specifying transcription factor and PET1 is a differentiation transcription factor important for driving the serotonergic phenotype and generally considered a cell identity marker for serotonergic neurons. These *r2Hoxa2-Pet1* neurons reside mainly in the median raphe (also referred to as prepontine raphe) and as a population collectively send large beaded axonal projections to key reward- and memory-relevant brain structures, namely the medial prefrontal cortex (enriched in deep layers), hippocampus, basolateral amygdala, ventral tegmental area, and substantia nigra, among other forebrain regions (Bang et al., 2012). The other subtype, referred to as *Drd1a-Pet1* neurons, originates within the isthmus/rhombomere 1 progenitor domain (along with other molecularly distinct serotonergic neurons) and expresses the transcription factor Engrailed1 (EN1) and the type 1a dopamine receptor during embryonic development and perinatally (Niederkofler et al., 2016). The cell bodies of this subtype, denoted as *Drd1a-Pet1* neurons, reside mainly in the dorsal raphe and, as a group, project morphologically fine axons to the medial prefrontal cortex (enriched in superficial and middle layers), CA1 hippocampus, nucleus accumbens shell, ventral tegmental area and substantia nigra, among other forebrain and midbrain regions (Niederkofler et al., 2016).

The *r2Hoxa2-Pet1* and *Drd1a-Pet1* neuron subtypes also manifest distinct organismal behavioral phenotypes upon in vivo suppression of neurotransmitter exocytosis (Kim et al., 2009; Niederkofler et al., 2016). Such silencing of *r2Hoxa2-Pet1* neurons enhanced prepulse inhibition of acoustic startle and social perseverative behavior without influencing horizontal locomotor activity, intra-species aggression, or social recognition (Kim et al., 2009; Okaty et al., 2015; Teissier et al., 2015; Niederkofler et al., 2016). By contrast, silencing *Drd1a-Pet1* neurons escalated intra-species aggression and caused social interaction deficits as compared with controls, without influencing horizontal locomotor activity, spatial or operant learning and memory, and depression- or anxiety-like behaviors (Niederkofler et al., 2016).

In the present study, conditional intersectional genetics was applied to drive the expression of the synthetic inhibitory DREADD (designer receptor exclusively activated by designer drug) receptor hM_4Di (Di), a Gi/o protein–coupled receptor activated by the synthetic ligand clozapine-N-oxide (CNO) (Armbruster et al., 2007), in *r2Hoxa2-Pet1* or *Drd1a-Pet1* neurons. This approach was previously validated in *Pet1*-raphe neurons using the same transgenic mouse lines (noting that *Drd1a-Pet1* falls within the published *En1-Pet1* group), demonstrating raphe inhibition in vivo 30 min after CNO injection as measured by in vivo electrophysiological recordings as well as by decreased FOS expression at 2 h post CNO administration (Teissier et al., 2015), by CNO-Di-triggered organismal behavioral and physiological phenotypes (Ray et al., 2011; Brust et al., 2014; Teissier et al., 2015; Dosumu-Johnson et al., 2018), and in brain slices by the decreased firing of *Pet1* neurons within seconds of CNO bath exposure (Ray et al., 2011; Brust et al., 2014). Based on distinct phenotypes associated with exocytic inhibition of *r2Hoxa2-Pet1* or *Drd1a-Pet1* neurons (Niederkofler et al., 2016; Okaty et al., 2015), along with their differential innervation profiles to various reward- and memory-relevant areas, we hypothesized that inhibition of *r2Hoxa2-Pet1* or *Drd1a-Pet1* neurons would have differential effects on cocaine CPP: *r2Hoxa2-Pet1* neurons would impact the memory aspect of cocaine CPP, whereas *Drd1a-Pet1* neurons would impact the reward aspect of cocaine CPP.

Material and methods

Animals

Adult male mice on a C57BL/6J background were used to study the expression and development of cocaine CPP, including foundational work determining behaviorally active doses of cocaine and assay-neutral doses of CNO. Triple

transgenics, abbreviated *r2Hoxa2-Pet1-Di* (for *r2Hoxa::cre*, *Pet::Flpe*, *RC::FPDi*) and *Drd1a-Pet1-Di* (for *Drd1a::cre*, *Pet::Flpe*, *RC::FPDi*), and their sibling controls were imported directly from the mouse breeding facility at Harvard Medical School ($N = 150$) or were generated at the Boston University mouse breeding facility after rederivation by embryo transfer ($N = 157$). Intersectional Di expression and function selectively in these distinct subtypes of $Pet1^{+}$ serotonergic neurons is summarized in Fig. S1 and has been previously established for these drivers (*r2Hoxa::cre*, *Drd1a::cre*, *Pet::Flpe*) and the intersectional conditional *Di* transgene knocked into the *ROSA26* locus and denoted *RC::FPDi* (Jensen et al., 2008; Brust et al., 2014; Teissier et al., 2015; Okaty et al., 2015; Niederkofler et al., 2016; Ray et al., 2011; Dosumu-Johnson et al., 2018). Mice were kept on a 12-h light schedule (7:00 am on, 7:00 pm off) and had ad libitum access to food and water. All housing and behavioral procedures were approved by the respective Institutional Animal Care and Use Committee (IACUC) at Harvard Medical School and Boston University.

Conditioned place preference assay

CPP procedures were performed in 3-compartment chambers (Model ENV 3013, Med Associates, St. Albans, VT) enclosed in ventilated sound-attenuating cubicles. The side compartments had distinct visual, tactile, and odor cues and were adjacent to a smaller gray center compartment (starting location). The right side was black and contained a rod floor and cedar bedding; the left side was white and contained a mesh floor and pine bedding. The gray center compartment had a smooth floor, with no associated odor cue. A series of photobeam sensors were located throughout the CPP chamber to measure horizontal locomotor activity. All testing took place during the light phase of the light-dark cycle. Mice received 3 daily pre-conditioning preference tests (all compartments available for 15 min after a 5-min hold time in the center compartment), with the last test session used to establish baseline preference for the compartments to be paired with cocaine (S^{+}) and saline (S^{-}). An unbiased conditioning procedure was used whereby half of each group randomly received cocaine in the black compartment and the other half in the white compartment. In the few cases ($n = 11$ sibling controls, $n = 2$ *r2Hoxa2-Pet1-Di*, and $n = 2$ *Drd1a-Pet1-Di*) whereby the randomly assigned cocaine compartment was associated with a strong pre-conditioning side preference (i.e., spending > 65% of time in that compartment), mice were conditioned with cocaine in the non-preferred compartment. Mice experienced 8 daily conditioning sessions (30 min each; confined to the assigned compartment) with alternating intraperitoneal (i.p.) injections of cocaine and saline (counterbalanced injection order). Following the conditioning period, 6 daily post-conditioning preference tests (all compartments available for 15 min after a 5-min hold time in the center compartment) were conducted. A preference score was calculated as the difference between time spent (s) in the cocaine compartment after conditioning relative to before conditioning (Carr et al., 1989). In addition, horizontal locomotor activity (total counts of 3 consecutive photobeam breaks in the chamber) was recorded for each session and served as an internal behavioral control measure.

Pilot experiment: dose-related effects of cocaine in the CPP assay

Pilot experiments were conducted in triple transgenic and sibling control mice to determine behaviorally active doses of cocaine for further study. Mice received either 1.0 ($n = 12$ *r2Hoxa2-Pet1-Di* with $n = 12$ sibling controls and $n = 10$ *Drd1a-Pet1-Di* with $n = 7$ sibling controls), 3.0 ($n = 17$ *r2Hoxa2-Pet1-Di* with $n = 15$ sibling controls and $n = 7$ *Drd1a-Pet1-Di* with $n = 10$ sibling controls), 10.0 ($n = 10$ *r2Hoxa2-Pet1-Di* with $n = 12$ sibling controls and $n = 12$ *Drd1a-Pet1-Di* with $n = 17$ sibling controls) or 17.8 ($n = 9$ *r2Hoxa2-Pet1-Di* with $n = 10$ sibling controls and $n = 17$ *Drd1a-Pet1-Di* with $n = 11$ sibling controls) mg/kg cocaine immediately prior to cocaine (S^{+}) conditioning sessions and an equal volume of sterile 0.9% saline (10 ml/kg) immediately prior to saline (S^{-}) conditioning sessions, with mice experiencing a total of 8 daily conditioning sessions with alternating intraperitoneal (i.p.) injections of cocaine and saline. Following conditioning, mice received saline injection (i.p.) 30 min before a post-conditioning session to measure cocaine CPP. Based on this study (see Figs. 2 and 7 below), 10.0 and 17.8 mg/kg cocaine were selected for further study to assess the effects of CNO-mediated inhibition of *r2Hoxa2-Pet1* and *Drd1a-Pet1* neurons on the expression and development of cocaine CPP.

Pilot experiment: CNO dose neutrality for cocaine CPP

A pilot experiment in wild-type C57BL/6J male mice ($n = 4$), the majority genetic background of our experimental transgenics determined the dose-related effects of CNO administration itself, absent Di expression, and signaling. Mice were conditioned, as described above, with alternating i.p. injections of 10.0 mg/kg cocaine in the S^{+} compartment and 10 ml/kg saline in the S^{-} compartment. Following the conditioning period, cocaine reward and locomotor activity were measured for 15 min, starting 30 min after CNO injection (1.0, 5.0, and 10 mg/kg administered i.p. in a random order in individual mice on post-conditioning sessions 2, 4, and 6), or after saline injection instead of CNO (on post-conditioning sessions 1, 3, and 5). CNO injection (1.0–10.0 mg/kg) did not significantly modify cocaine CPP expression or locomotor activity relative to saline injection, indicating that 10.0 mg/kg CNO is inert to these queried behaviors in C57BL/6J mice (Fig. S2). Based on the results of this study, 10 mg/kg CNO was selected to assess the effects of CNO-silencing of *r2Hoxa2-Pet1* and *Drd1a-*

Pet1 neurons on the expression and development of cocaine CPP; a CNO dose in line with our prior work establishing functional inhibition using this same *RC::FPDi* transgenic coupled with the *Pet1::Flpe* and Cre drivers (Jensen et al., 2008; Brust et al., 2014; Teissier et al., 2015; Okaty et al., 2015; Niederkofler et al., 2016; Ray et al., 2011; Dosumu-Johnson et al., 2018).

Expression of cocaine CPP

The experimental design to study the expression of cocaine CPP is illustrated in the top half of Fig. 1. Mice were conditioned with 10.0 or 17.8 mg/kg cocaine. During the post-conditioning period, mice received saline injection (i.p.) 30 min before post-conditioning session 1 to establish baseline cocaine preference in mice with uninhibited 5-HT neurons. Mice then received 10.0-mg/kg CNO injection (i.p.) 30 min prior to post-conditioning session 2 to determine the effects of inhibiting the specific *Pet1* neuron subtype on the expression of cocaine CPP. CNO was not given during conditioning and the remaining 4 post-conditioning sessions (sessions 3–6) were conducted without CNO or saline injections to determine the persistence of cocaine CPP in the cocaine-free state. Preference scores and locomotor activity for each session were calculated, as described above. CPP expression testing was initially conducted probing *r2Hoxa2-Pet1* neurons (experiment 1; $n = 10$ *r2Hoxa2-Pet1-Di* mice and $n = 12$ sibling controls for the 10.0-mg/kg cocaine dose; $n = 9$ *r2Hoxa2-Pet1-Di* mice and $n = 10$ sibling controls for the 17.8-mg/kg cocaine dose) and then repeated to probe *Drd1a-Pet1* neurons (experiment 2; $n = 12$ *Drd1a-Pet1-Di* mice and $n = 17$ sibling controls for the 10.0-mg/kg cocaine dose; $n = 17$ *Drd1a-Pet1-Di* mice and $n = 11$ sibling controls for the 17.8-mg/kg cocaine dose). Note that these were the same animals conditioned with 10.0 and 17.8 mg/kg cocaine in the dose-response pilot experiments.

Development of cocaine CPP

The experimental design to study the development of cocaine CPP is illustrated in the bottom half of Fig. 1. A different set of mice were conditioned with 10.0 or 17.8 mg/kg cocaine and received 10.0-mg/kg CNO injection 30 min prior to each of the cocaine (S^+) and saline (S^-) conditioning sessions. All CNO was only given during conditioning sessions. No injections were given during the 6-session post-conditioning period to explore cocaine place conditioning and its persistence in the cocaine-free state. Preference scores and locomotor activity for each session were calculated, as described above. CPP development testing was initially conducted probing *r2Hoxa2-Pet1* neurons (experiment 1; $n = 14$ *r2Hoxa2-Pet1-Di* mice and $n = 10$ sibling controls for the 10.0-mg/kg cocaine dose; $n = 12$ *r2Hoxa2-Pet1-Di* mice and $n = 10$ sibling controls for the 17.8-mg/kg cocaine dose) and then repeated to probe *Drd1a-Pet1* neurons (experiment 2; $n = 15$ *Drd1a-Pet1-Di* mice and $n = 14$ sibling controls for the 10.0-mg/kg cocaine dose; $n = 12$ *Drd1a-Pet1-Di* mice and $n = 7$ sibling controls for the 17.8-mg/kg cocaine dose).

Statistics

Data from 283 mice were analyzed in the above-described experiments involving transgenic animals. To compare cocaine dose-response functions during the conditioning period, locomotor activity counts for the 30-min sessions were averaged across the four cocaine sessions and the four saline sessions for each mouse prior to analysis. These data were analyzed by

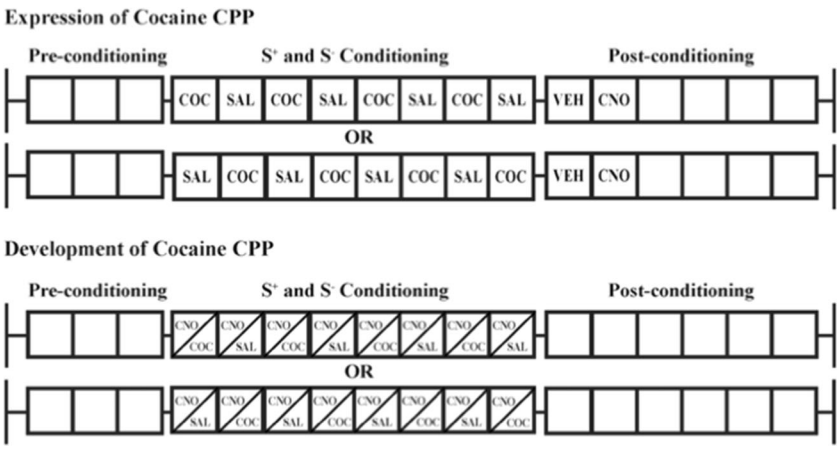


Fig. 1 General experimental design to assess the effects of CNO-Di-triggered inhibition of *Pet1* neurons on the expression (top) and development (bottom) of cocaine-conditioned place preference (CPP). S^+ refers to cocaine conditioning sessions and S^- to saline conditioning sessions. Boxes indicate the number of sessions conducted for the pre-conditioning (3), conditioning (8), and post-conditioning (6) phases of the experiments. Saline (SAL) and clozapine-N-oxide (CNO) were administered 30 min prior to post-conditioning sessions 1 and 2 during CPP expression testing. During CPP development testing, CNO was administered 30 min prior to cocaine and saline conditioning sessions

two-factor repeated measures ANOVA (dose-response pilot experiments) or three-factor repeated measures ANOVA (expression and development tests). To compare cocaine dose-response functions at the start of the post-conditioning period, preference scores and 15-min locomotor activity counts were analyzed for session 1 (dose-response pilot experiment) or for sessions 1 and 2 (expression and development tests) by one-factor ANOVA or three-factor repeated measures ANOVA, respectively. To determine the time course of changes in cocaine preference scores and locomotor activity after session 2, analysis of post-conditioning sessions 2–6 was performed for each cocaine dose using two-factor repeated measures ANOVA (expression and development tests). The Tukey procedure was used for all post hoc testing. There were an additional 25 mice across experiments with outlying preference scores (more than 2 standard deviations above or below their respective group means), and thus, data from these mice ($n = 2$ *r2Hoxa2-Pet1-Di* and $n = 8$ sibling control mice in experiment 1; $n = 8$ *Drd1a-Pet1-Di* and $n = 7$ sibling control mice in experiment 2) were not included in this report.

Results

Experiment 1. *r2Hoxa2-Pet1-Di* and sibling control mice

Dose-related effects of cocaine in the CPP assay

In the pilot experiment (absent CNO), cocaine produced dose-related effects on locomotor activity and cocaine preference in *r2Hoxa2-Pet1-Di* and sibling control mice. Locomotor activity (Fig. 2a) was greater during conditioning with 3.0, 10.0, and 17.8 mg/kg cocaine compared with the corresponding saline conditioning session ($ps < 0.03$), with 10.0 and 17.8 mg/kg cocaine compared with 1.0 and 3.0 mg/kg cocaine ($ps < 0.001$), and with 17.8 compared with 10.0 mg/kg cocaine ($p < 0.001$), based on the cocaine dose × conditioning session type interaction ($F[3,89] = 167.3$, $p < 0.001$). After conditioning (Fig. 2b), 10.0 and 17.8 mg/kg cocaine produced higher preference scores than 1.0 and 3.0 mg/kg cocaine ($ps < 0.02$), in follow-up to the cocaine dose main effect ($F[3,89] = 11.4$, $p < 0.001$). Although there appear to be higher preference scores in *r2Hoxa2-Pet1-Di* mice than sibling controls for the 1.0 and 3.0 cocaine conditioning doses, the genotype main effect ($p < 0.11$) and the genotype × cocaine dose interaction ($p < 0.18$) were not significant. Consequent to this pilot experiment, we selected 10.0 and 17.8 mg/kg cocaine as behaviorally active doses to assess the effects of CNO-mediated inhibition of *r2Hoxa2-Pet1* neurons on the expression and development of cocaine CPP.

CPP expression testing

Cocaine reward During the initial two post-conditioning preference tests conducted in the cocaine-free state, there were lower cocaine preference scores after session

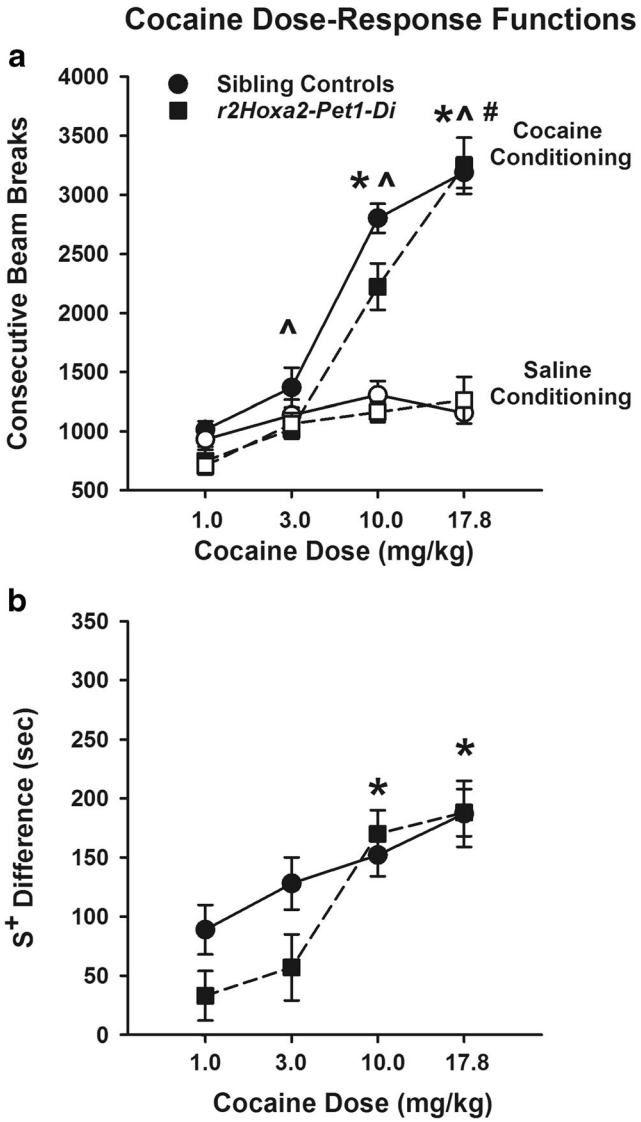


Fig. 2 Cocaine dose-response functions in *r2Hoxa2-Pet1-Di* and sibling control mice. Values are the mean ± s.e.m. consecutive photobeam breaks (locomotor activity) averaged in individual mice over the four 30-min conditioning sessions with cocaine vs. saline (**a**) and the S^+ difference scores (cocaine preference) following conditioning doses of 1.0, 3.0, 10.0, and 17.8 mg/kg cocaine (**b**). In **a**, $*p < 0.001$ comparing 10.0 and 17.8 mg/kg cocaine with 1.0 and 3.0 mg/kg cocaine across genotypes. $\#p < 0.001$ comparing 17.8 mg/kg cocaine with 10.0 mg/kg cocaine across genotypes. $\wedge p < 0.03$ comparing 3.0, 10.0, and 17.8 mg/kg cocaine with saline across genotypes. In **b**, $*p < 0.02$ comparing 10.0- and 17.8-mg/kg cocaine conditioning doses with 1.0- and 3.0-mg/kg conditioning doses across genotypes. $N = 12$ sibling controls and 12 *r2Hoxa2-Pet1-Di* mice for 1.0 mg/kg cocaine; $N = 15$ sibling controls and 17 *r2Hoxa2-Pet1-Di* mice for 3.0 mg/kg cocaine; $N = 12$ sibling controls and 10 *r2Hoxa2-Pet1-Di* mice for 10.0 mg/kg cocaine; and $N = 10$ sibling controls and 9 *r2Hoxa2-Pet1-Di* mice for 17.8 mg/kg cocaine

2 (acute CNO injection) than session 1 (acute saline injection), in follow-up to the session number main effect (F[1,37] = 8.0, $p < 0.01$). Preference scores did not vary significantly by cocaine dose or genotype (Fig. 3a). In the time course analysis of post-conditioning sessions 2–6, preference scores did not vary significantly across sessions or genotypes for either cocaine conditioning dose (Fig. 3b).

Locomotor activity During conditioning sessions (Fig. 4a), locomotor activity was greater for each dose of cocaine compared with that of saline ($ps < 0.001$) and with 17.8 compared with 10.0 mg/kg cocaine ($p < 0.001$), based on the cocaine dose × conditioning session type interaction

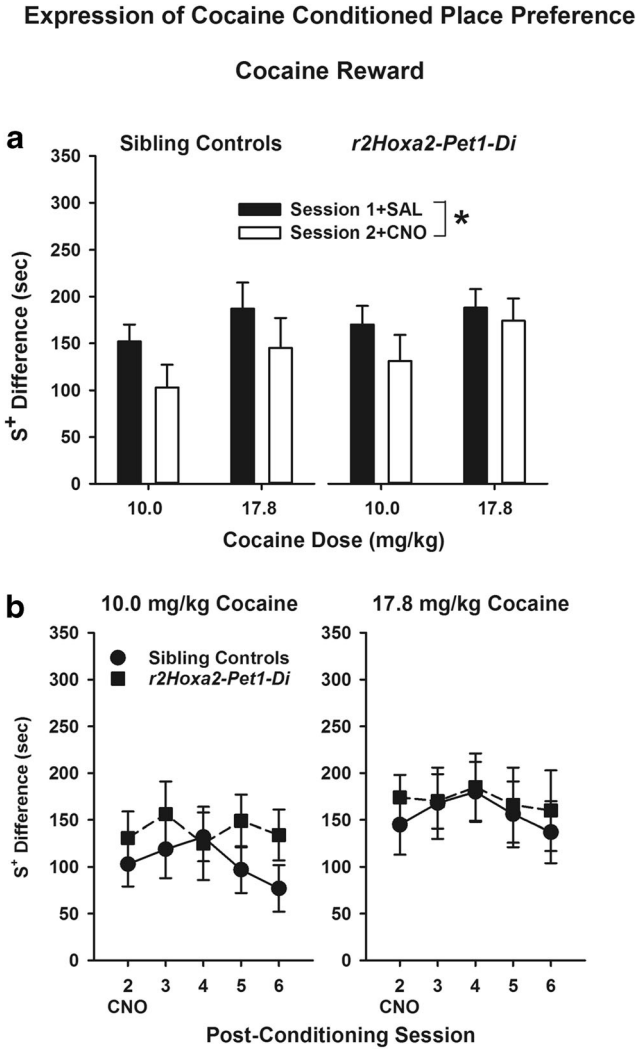


Fig. 3 CPP expression testing in *r2Hoxa2-Pet1-Di* and sibling control mice: Cocaine preference. Values are the mean ± s.e.m. S^+ difference scores during the first and second post-conditioning sessions (**a**) and the time course of changes in S^+ difference scores during post-conditioning sessions 2–6 (**b**). Mice received a SAL injection 30 min prior to post-conditioning session 1 and a 10.0-mg/kg CNO injection 30 min prior to post-conditioning session 2, with no injections during post-conditioning sessions 3–6. In **a**, $*p < 0.01$ comparing session 1 with session 2 across genotypes and cocaine conditioning doses. $N = 12$ sibling controls and 10 *r2Hoxa2-Pet1-Di* mice for 10.0 mg/kg cocaine and $N = 10$ sibling controls and 9 *r2Hoxa2-Pet1-Di* mice for 17.8 mg/kg cocaine

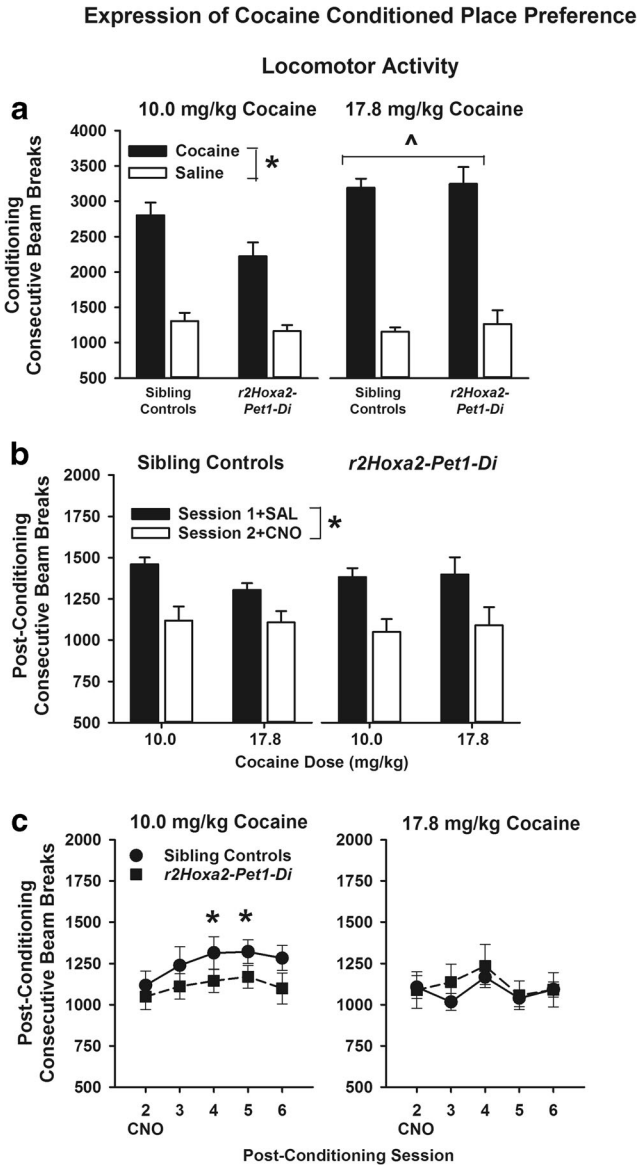


Fig. 4 CPP expression testing in *r2Hoxa2-Pet1-Di* and sibling control mice: Locomotor activity. Values are the mean ± s.e.m. consecutive photobeam breaks averaged in individual mice over the four 30-min conditioning sessions with cocaine vs. saline (**a**), during the first and second post-conditioning sessions (**b**), and during post-conditioning sessions 2–6 (**c**). Mice received a SAL injection 30 min prior to post-conditioning session 1 and a 10.0-mg/kg CNO injection 30 min prior to post-conditioning session 2, with no injections during post-conditioning sessions 3–6. In **a**, $*p < 0.001$ comparing cocaine conditioning with saline conditioning across genotypes and cocaine doses. $\wedge p < 0.001$ comparing 17.8 mg/kg cocaine with 10.0 mg/kg cocaine across genotypes. In **b**, $*p < 0.001$ comparing session 1 with session 2 across genotypes and cocaine conditioning doses. In (C), $*p < 0.03$ comparing session 4 and 5 with session 2 across genotypes for the 10.0-mg/kg cocaine conditioning dose. $N = 12$ sibling controls and 10 *r2Hoxa2-Pet1-Di* mice for 10.0 mg/kg cocaine and $N = 10$ sibling controls and 9 *r2Hoxa2-Pet1-Di* mice for 17.8 mg/kg cocaine

(F[1,37] = 23.3, $p < 0.001$). Locomotor activity did not vary significantly by genotype during conditioning. During the initial two post-conditioning preference tests conducted in the cocaine-free state (Fig. 4b), there was less locomotor activity after session 2 (acute CNO injection) than session 1 (acute saline injection), in follow-up to the session number main effect (F[1,37] = 78.1, $p < 0.001$). There were no significant differences due to cocaine dose or genotype. In the time course analysis of post-conditioning sessions 2–6 (Fig. 4c), there was greater locomotor activity during sessions 4 and 5 compared with that during session 2 (ps < 0.03) for the 10.0-mg/kg cocaine conditioning dose across genotypes, based on the session number main effect (F[4,80] = 3.3, $p < 0.01$). For the 17.8-mg/kg cocaine conditioning dose, there were no significant differences in locomotor activity across sessions 2–6 in either genotype.

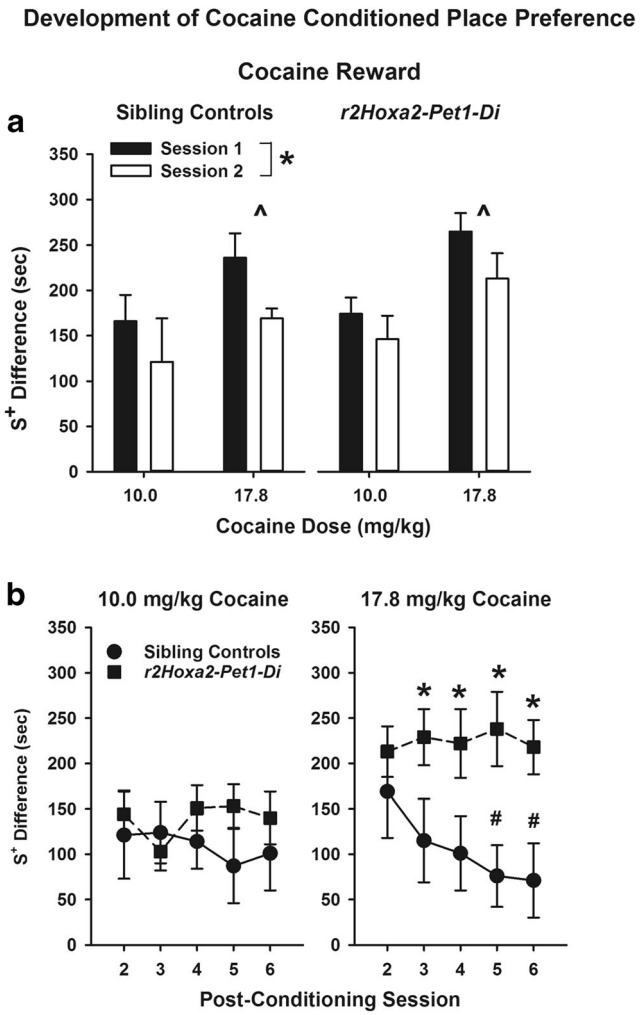


Fig. 5 CPP development testing in *r2Hoxa2-Pet1-Di* and sibling control mice: Cocaine preference. Values are the mean ± s.e.m. S^+ difference scores during the first and second post-conditioning sessions (**a**) and the time course of changes in S^+ difference scores during post-conditioning sessions 2–6. (**b**) Mice received CNO injections during conditioning sessions, with no injections during post-conditioning sessions 1–6. In **a**, *$p <$ 0.001 comparing session 1 with session 2 across genotypes and cocaine conditioning doses. ^$p < 0.02$ comparing the 17.8-mg/kg with the 10.0-mg/kg cocaine conditioning doses across genotypes and sessions. In (B), #$p < 0.004$ comparing sessions 5 and 6 with session 2 in sibling control mice for the 17.8-mg/kg cocaine conditioning dose. *$p < 0.04$ comparing sessions 3–6 in *r2Hoxa2-Pet1-Di* mice with sibling controls for the 17.8-mg/kg cocaine conditioning dose. N = 10 sibling controls and 14 *r2Hoxa2-Pet1-Di* mice for 10.0 mg/kg cocaine and N = 10 sibling controls and 12 *r2Hoxa2-Pet1-Di* mice for 17.8 mg/kg cocaine

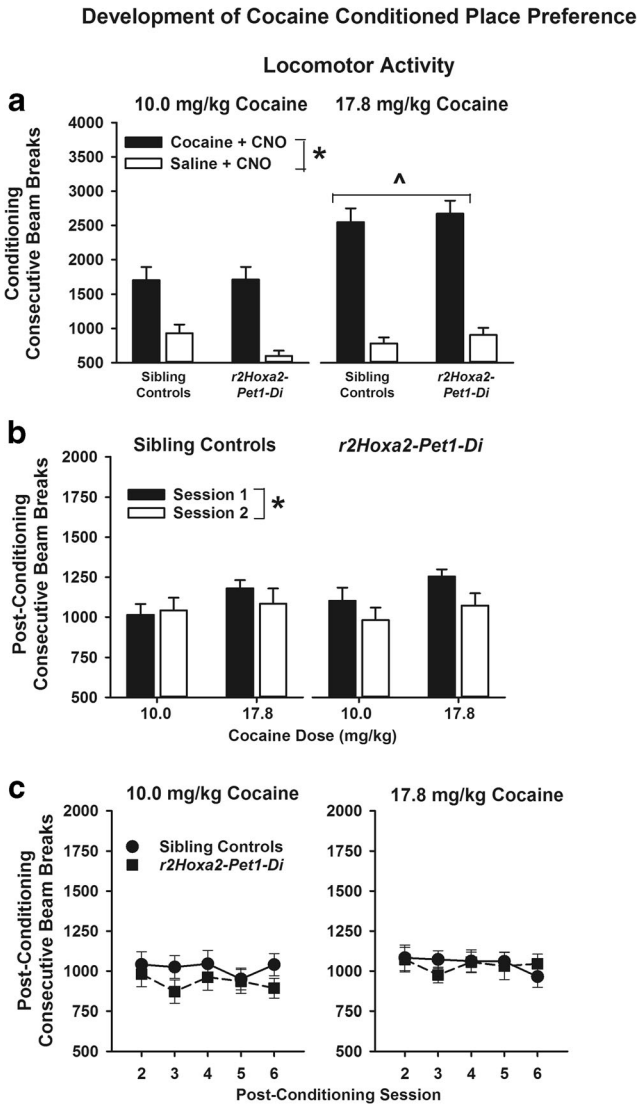


Fig. 6 CPP development testing in r2Hoxa2-Pet1-Di and control mice: Locomotor activity. Values are the mean ± s.e.m. consecutive photobeam breaks averaged in individual mice over the four 30-min conditioning sessions with cocaine vs. saline (**a**), during the first and second post-conditioning sessions (**b**), and during post-conditioning sessions 2–6 (**c**). Mice received CNO injections during conditioning sessions, with no injections during post-conditioning sessions 1–6. In **a**, *$p < 0.001$ comparing cocaine (+ CNO) conditioning sessions with saline (+ CNO) across genotypes and cocaine doses. ^$p < 0.001$ comparing 17.8 mg/kg cocaine (+ CNO) with 10.0 mg/kg cocaine (+ CNO) across genotypes. In (B), *$p < 0.02$ comparing session 1 with session 2 across genotypes and cocaine conditioning doses. N = 10 sibling controls and 14 *r2Hoxa2-Pet1-Di* mice for 10.0 mg/kg cocaine and N = 10 sibling controls and 12 *r2Hoxa2-Pet1-Di* mice for 17.8 mg/kg cocaine

CPP development testing

Cocaine reward During the initial two post-conditioning preference tests (cocaine-free and CNO-free states), cocaine preference scores were higher with the 17.8 compared with the 10.0-mg/kg conditioning dose and during post-conditioning session 1 compared with that during session 2, based on the main effects of cocaine dose (F[1,42] = 6.1, $p < 0.02$) and session number (F[1,42] = 12.9, $p < 0.001$) (Fig. 5a). Although CNO administration during conditioning did not influence the initial development of cocaine reward in *r2Hoxa2-Pet1-Di* mice, preference scores for 17.8 mg/kg cocaine were more persistently maintained in these mice over the post-conditioning period (Fig. 5b). In support of this, the time course analysis of post-conditioning sessions 2–6 performed for each cocaine conditioning dose revealed no significant factors for the 10.0-mg/kg cocaine conditioning dose, but a genotype × session number interaction (F[4,80] = 3.5, $p < 0.01$) for the 17.8-mg/kg cocaine conditioning dose. Sibling controls exhibited lower cocaine preference scores on sessions 5 and 6 compared with that on session 2 (ps < 0.004), whereas in *r2Hoxa2-Pet1-Di* mice, cocaine preference scores were not significantly different across sessions 2–6 and were significantly higher than sibling controls on post-conditioning sessions 3–6 (ps < 0.04).

Locomotor activity During conditioning sessions with 10.0 and 17.8 mg/kg cocaine (Fig. 6a), locomotor activity was greater during cocaine (+CNO) than saline (+CNO) conditioning ($p < 0.001$) and with 17.8 compared with 10.0 mg/kg cocaine ($p < 0.001$), based on the cocaine dose × conditioning session type interaction (F[1,42] = 22.7, $p < 0.001$). Locomotor activity did not vary significantly by genotype during conditioning. When mice were tested in the cocaine-free and CNO-free states on post-conditioning sessions 1 and 2, there was less locomotor activity during session 2 than session 1 (session number main effect; F[1,42] = 6.5, $p < 0.02$), but no significant differences due to cocaine dose or genotype (Fig. 6b). In the time course analysis of post-conditioning sessions 2–6, locomotor activity within sibling controls and *r2Hoxa2-Pet1-Di* mice did not significantly differ across sessions for either the 10.0 or 17.8-mg/kg cocaine conditioning dose (Fig. 6c).

Experiment 2. *Drd1a-Pet1-Di* and sibling control mice

Dose-related effects of cocaine in the CPP assay

In the pilot experiment, cocaine produced dose-dependent effects on locomotor activity and cocaine preference in *Drd1a-Pet1-Di* and sibling control mice. Locomotor activity (Fig. 7a) was greater during conditioning with 10.0 and 17.8 mg/kg cocaine compared with that during the corresponding saline conditioning session (ps < 0.001), with 10.0 and 17.8 mg/kg cocaine compared with 1.0 and 3.0 mg/kg cocaine (ps < 0.001), and with 17.8 compared with 10.0 mg/kg cocaine (p

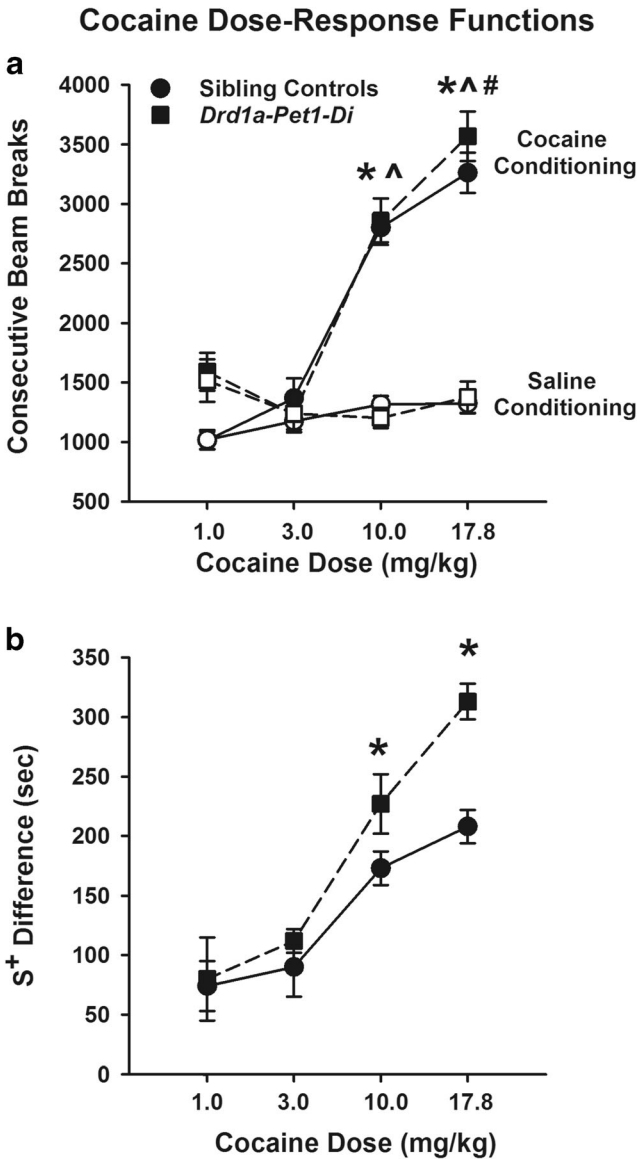


Fig. 7 Cocaine dose-response functions in *Drd1a-Pet1-Di* and sibling control mice. Values are the mean ± s.e.m. consecutive photobeam breaks averaged in individual mice over the four 30-min conditioning sessions with cocaine vs. saline (**a**) and the S^+ difference scores following conditioning doses of 1.0, 3.0, 10.0, and 17.8 mg/kg cocaine (**b**). In **a**, *$p <$ 0.001 comparing 10.0 and 17.8 mg/kg cocaine with 1.0 and 3.0 mg/kg cocaine across genotypes. #$p < 0.001$ comparing 17.8 mg/kg cocaine with 10.0 mg/kg cocaine across genotypes. ^$p < 0.001$ comparing 10.0 mg/kg and 17.8 mg/kg cocaine with saline across genotypes. In b, *$p < 0.001$ comparing 10.0- and 17.8-mg/kg cocaine conditioning doses with 1.0- and 3.0-mg/kg conditioning doses across genotypes. $N = 7$ sibling controls and 7 *Drd1a-Pet1-Di* mice for 1.0 mg/kg cocaine; $N = 10$ sibling controls and 7 *Drd1a-Pet1-Di* mice for 3.0 mg/kg cocaine; $N = 17$ sibling controls and 12 *Drd1a-Pet1-Di* mice for 10.0 mg/kg cocaine; and $N = 11$ sibling controls and 17 *Drd1a-Pet1-Di* mice for 17.8 mg/kg cocaine

< 0.001), based on the cocaine dose × conditioning session type interaction (F[3,83] = 68.5, $p < 0.001$). After conditioning (Fig. 7b), 10.0 and 17.8 mg/kg cocaine produced higher preference scores than 1.0 and 3.0 mg/kg cocaine (ps < 0.001) and 17.8 mg/kg cocaine produced a higher preference score than 10 mg/kg cocaine ($p < 0.01$), in follow-up to the cocaine dose main effect (F[3,83] = 31.6, $p < 0.001$). This again demonstrated that 10.0 and 17.8 mg/kg cocaine were behaviorally active doses and were selected to assess the effects of CNO-mediated inhibition of *Drd1a-Pet1* neurons on the expression and development of cocaine CPP. It should be noted that a main effect of genotype also was revealed for preference scores in *Drd1a-Pet1-Di* and sibling control mice (F[3,83] = 9.1, $p < 0.003$), and this aspect is described more fully in the next ANOVA analysis.

CPP expression testing

Cocaine reward During the initial two post-conditioning preference tests (cocaine-free state with an acute saline then CNO injection), the preference scores were higher after 17.8 mg/kg than 10.0 mg/kg cocaine (main effect of cocaine dose; (F[1,53] = 13.9, $p < 0.001$)). However, based on the genotype × session number interaction (F[1,53] = 3.9, $p < 0.05$), *Drd1a-Pet1-Di* mice exhibited higher preference scores than sibling controls on session 1 for the 10.0- and 17.8-mg/kg cocaine conditioning doses ($p <$ 0.001), whereas on session 2, CNO-induced *Drd1a-Pet1* neuron inhibition led to no significant differences in preference scores between the two genotypes for both cocaine conditioning doses (Fig. 8a). In the time course analysis of post-conditioning sessions 2–6 performed for each cocaine conditioning dose, a rebound from this reduced cocaine preference was revealed in *Drd1a-Pet1-Di* mice 48 h following CNO administration (Fig. 8b). For the 10-mg/kg cocaine conditioning dose, *Drd1a-Pet-Di* mice exhibited higher preference scores during post-conditioning sessions 4–6 compared with that during session 2 (the CNO treatment session) and compared with that during post-conditioning sessions 4–6 in sibling controls (ps < 0.04), based on the genotype × session number interaction (F[4, 108] = 3.7, $p < 0.01$). For the 17.8-mg/kg cocaine conditioning dose, genotype was significant factor (F[1, 26] = 6.7, $p < 0.02$) wherein *Drd1a-Pet1-Di* mice had higher preference scores than sibling controls overall, but additional Tukey comparisons clarified that the differences between *Drd1a-Pet1-Di* mice and sibling controls were significant for post-conditioning sessions 4–6 (ps < 0.01) but not post-conditioning sessions 2 and 3.

Locomotor activity During conditioning sessions (Fig. 9a), locomotor activity was greater for each dose of cocaine compared with saline (ps < 0.001) and with 17.8 compared with 10.0 mg/kg cocaine ($p < 0.001$), based on the cocaine dose × conditioning session type interaction (F[1,53] = 8.0, $p < 0.01$). Locomotor activity did not vary significantly by genotype during conditioning. When mice were tested in the cocaine-free state during post-conditioning sessions 1 and 2 (Fig. 9b), there was less locomotor activity after session 2 (CNO administration) than session 1 (saline administration) for each

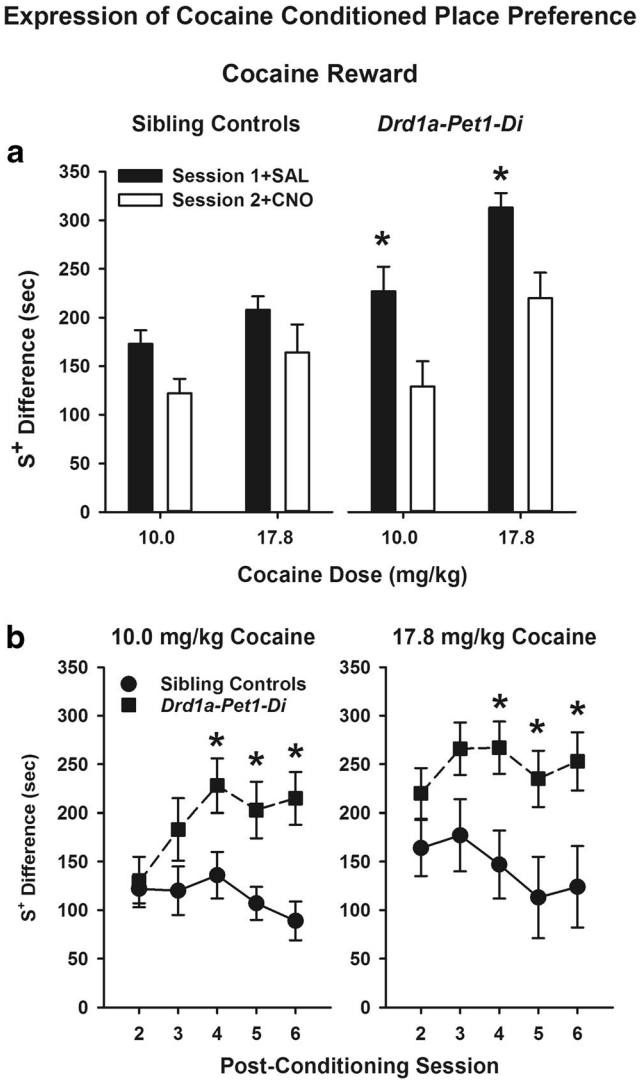
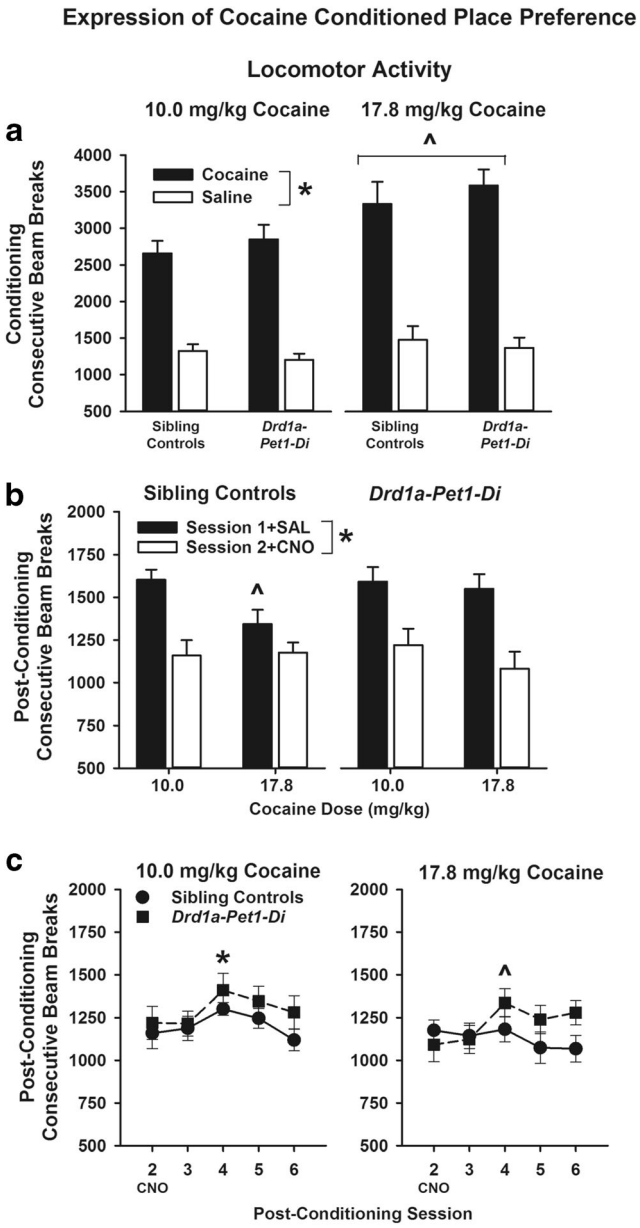


Fig. 8 CPP expression testing in *Drd1a-Pet1-Di* and sibling control mice: Cocaine preference. Values are the mean ± s.e.m. S^+ difference scores during the first and second post-conditioning sessions (**a**) and the time course of changes in S^+ difference scores during post-conditioning sessions 2–6. (**b**) Mice received a saline (SAL) injection 30 min prior to post-conditioning session 1 and a 10.0-mg/kg CNO injection 30 min prior to post-conditioning session 2, with no injections during post-conditioning sessions 3–6. In **a**, *$p < 0.001$ comparing the 10.0-mg/kg and 17.8-mg/kg cocaine conditioning doses in *Drd1a-Pet1-Di* mice with sibling control mice on session 1. Genotypes did not differ on session 2. In **b**, *$p < 0.04$ comparing *Drd1a-Pet1-Di* mice with sibling control mice for both the 10.0- and 17.8-mg/kg cocaine conditioning doses. $N = 17$ sibling controls and 12 *Drd1a-Pet1-Di* mice for 10.0 mg/kg cocaine and $N = 11$ sibling controls and 17 *Drd1a-Pet1-Di* mice for 17.8 mg/kg cocaine

genotype and cocaine conditioning dose (*p*s < 0.001) in addition to less locomotor activity after 17.8 than 10.0 mg/kg cocaine conditioning in sibling controls ($p < 0.03$) during session 1, based on the genotype × cocaine dose × session number interaction ($F[1,53] = 4.2$, $p < 0.047$). In the time course analysis of post-conditioning sessions 2–6 performed for each cocaine conditioning dose (Fig. 9c), there was greater locomotor activity on session 4 compared with that on sessions 2, 3, and 6 across genotypes (*p*s < 0.005) for the 10-mg/kg cocaine conditioning dose (session number main effect; $F[4, 108] = 5.8$, $p < 0.001$). For the 17.8-mg/kg cocaine conditioning dose, there was greater locomotor activity on session 4 compared with that on sessions 2 and 3 only in *Drd1a-Pet1-Di* mice (*p*s < 0.01), in follow-up to the genotype × session number interaction ($F[4, 104] = 3.4$, $p < 0.01$).



◀ **Fig. 9** CPP expression testing in *Drd1a-Pet1-Di* and sibling control mice: Locomotor activity. Values are the mean ± s.e.m. consecutive photobeam breaks averaged in individual mice over the four 30-min conditioning sessions with cocaine vs. saline (**a**), during the first and second post-conditioning sessions (**b**), and during post-conditioning sessions 2–6 (**c**). Mice received a saline (SAL) injection 30 min prior to post-conditioning session 1 and a 10.0-mg/kg CNO injection 30 min prior to post-conditioning session 2, with no injections during post-conditioning sessions 3–6. In **a**, $*p < 0.001$ comparing cocaine conditioning with saline conditioning across genotypes and cocaine doses. $\wedge p < 0.001$ comparing 17.8 mg/kg cocaine with 10.0 mg/kg cocaine across genotypes. In **b**, $*p < 0.001$ comparing session 1 with session 2 across genotypes and cocaine conditioning doses. $\wedge p < 0.03$ comparing the 17.8- with the 10.0-mg/kg cocaine conditioning dose in sibling control mice on session 1. In **c**, $*p < 0.005$ comparing session 4 with sessions 2, 3, and 6 in *Drd1a-Pet1-Di* mice and sibling controls after the 10.0-mg/kg cocaine conditioning dose. $\wedge p < 0.01$ comparing session 4 with sessions 2 and 3 in *Drd1a-Pet1-Di* mice after the 17.8-mg/kg cocaine conditioning dose. $N = 17$ sibling controls and 12 *Drd1a-Pet1-Di* mice for 10.0 mg/kg cocaine and $N = 11$ sibling controls and 17 *Drd1a-Pet1-Di* mice for 17.8 mg/kg cocaine

CPP development testing

Cocaine reward During the initial two post-conditioning preference tests (cocaine-free and CNO-free state), there were lower preference scores during post-conditioning session 2 compared with that during session 1 (Fig. 10a) across cocaine doses and genotypes (session number main effect; $F[1,44] = 19.6$, $p < 0.001$). The time course analysis of post-conditioning sessions 2–6 (Fig. 10b) revealed that both *Drd1a-Pet1-Di* mice and sibling controls further extinguished cocaine preference for the 17.8-mg/kg conditioning dose (main effect of session number; $F[4,68] = 4.1$, $p < 0.005$), with lower preference scores on session 6 compared with that on sessions 2 ($p < 0.007$) and 4 ($p < 0.049$).

Locomotor activity During conditioning sessions with 10.0 and 17.8 mg/kg cocaine (Fig. 11a), locomotor activity was greater during cocaine (+CNO) than saline (+CNO) conditioning ($p < 0.001$) and with 17.8 compared with 10.0 mg/kg cocaine ($p < 0.04$), based on the cocaine dose × conditioning session type interaction ($F[1,44] = 6.9$, $p < 0.05$). Locomotor activity did not vary significantly by genotype during conditioning. When mice were tested in the cocaine-free and CNO-free states on post-conditioning sessions 1 and 2 (Fig. 11b), there was less locomotor activity during session 2 than session 1 ($p < 0.001$) across cocaine doses and genotypes (session number main effect; $F[1,44] = 14.9$, $p < 0.001$). In the time course analysis of post-conditioning sessions 2–6 (Fig. 11c), there was less locomotor activity on session 6 than session 4 ($p < 0.001$)

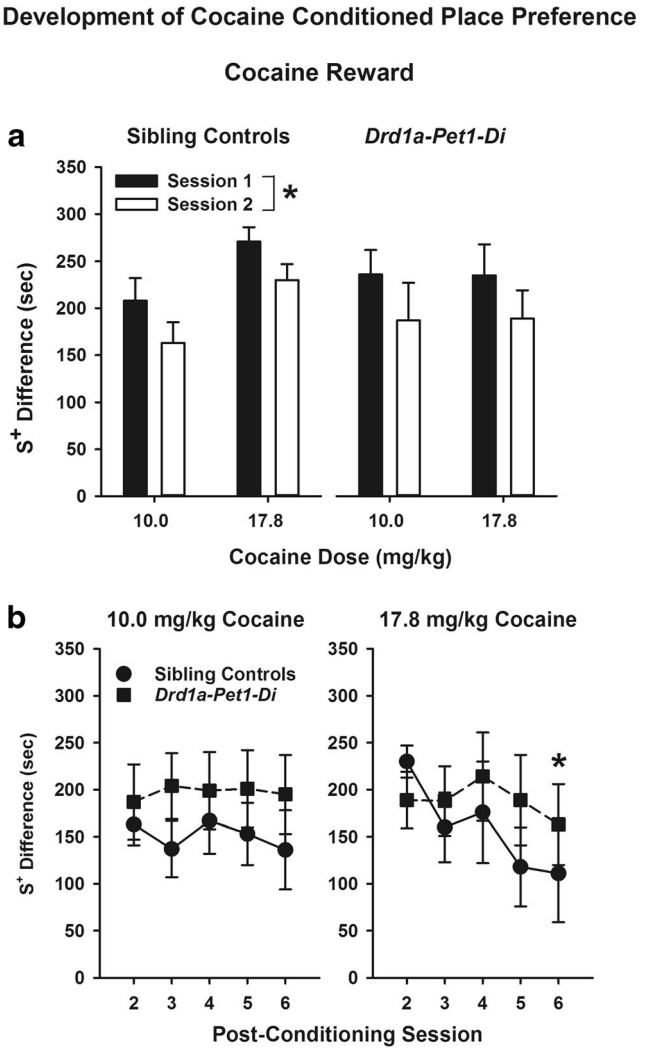


Fig. 10 CPP development testing in *Drd1a-Pet1-Di* and sibling control mice: Cocaine preference. Values are the mean ± s.e.m. S^+ difference scores during the first and second post-conditioning sessions (a) and the time course of changes in S^+ difference scores during post-conditioning sessions 2–6 (b). Mice received CNO injections during conditioning sessions, with no injections during post-conditioning sessions 1–6. In a, $*p < 0.001$ comparing session 1 with session 2 across genotypes and cocaine conditioning doses. In b, $*p < 0.049$ comparing session 6 with sessions 2 and 4 across genotypes. $N = 14$ sibling controls and 15 *Drd1a-Pet1-Di* mice for 10.0 mg/kg cocaine and $N = 7$ sibling controls and 12 *Drd1a-Pet1-Di* mice for 17.8 mg/kg cocaine

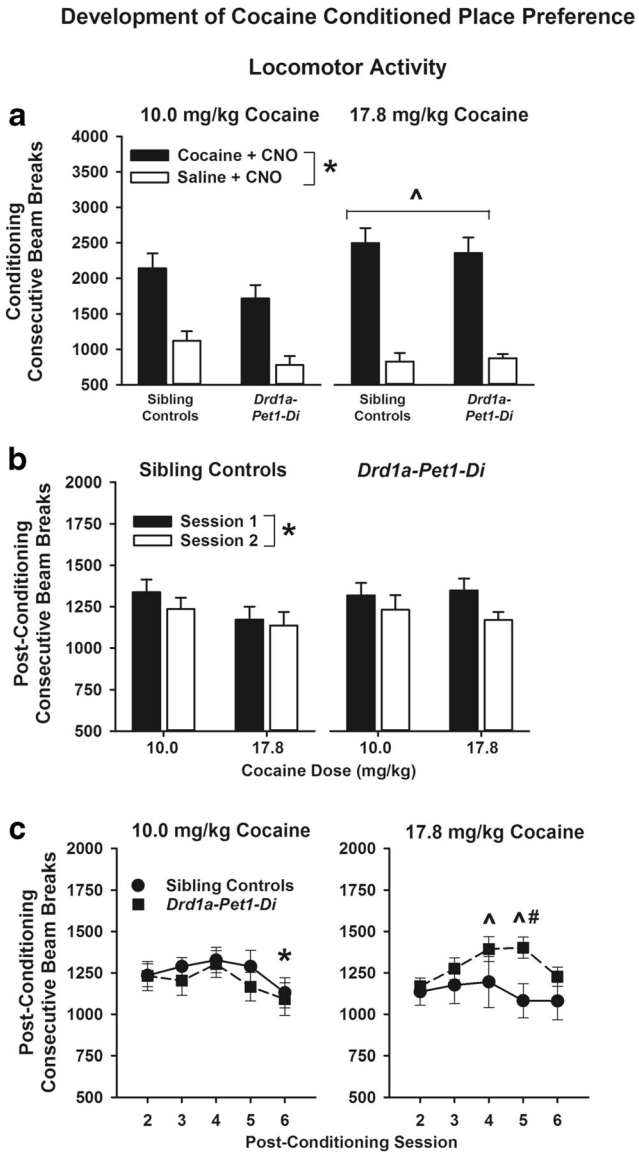


Fig. 11 CPP development testing in *Drd1a-Pet1-Di* and sibling control mice: Locomotor activity. Values are the mean ± s.e.m. consecutive photobeam breaks averaged in individual mice over the four 30-min conditioning sessions with cocaine vs. saline (**a**), during the first and second post-conditioning sessions (**b**), and during post-conditioning sessions 2–6 (**c**). Mice received CNO injections during conditioning sessions, with no injections during post-conditioning sessions 1–6. In **a**, $*p < 0.001$ comparing cocaine (+ CNO) conditioning with saline (+ CNO) conditioning across genotypes and cocaine doses. $^p < 0.04$ comparing 17.8 mg/kg cocaine (+ CNO) with 10.0 mg/kg cocaine (+ CNO) across genotypes. In **b**, $*p < 0.001$ comparing session 1 with session 2 across genotypes and cocaine conditioning doses. In **c**, $*p < 0.001$ comparing session 6 with session 4 across genotypes after conditioning with the 10.0-mg/kg cocaine dose. $^p < 0.04$ comparing sessions 4 and 5 with sessions 2 and 6 in *Drd1a-Pet1-Di* mice after the 17.8-mg/kg cocaine conditioning dose. $\#p < 0.01$ comparing *Drd1a-Pet1-Di* mice with sibling control mice on session 5 after the 17.8-mg/kg cocaine conditioning dose. $N = 14$ sibling controls and 15 *Drd1a-Pet1-Di* mice for 10.0 mg/kg cocaine and $N = 7$ sibling controls and 12 *Drd1a-Pet1-Di* mice for 17.8 mg/kg cocaine

across genotypes for the 10.0-mg/kg cocaine conditioning dose (session number main effect; $F[4, 108] = 4.4$, $p < 0.002$). For the 17.8-mg/kg conditioning dose, there was greater locomotor activity on sessions 4 and 5 than sessions 2 and 6 in *Drd1a-Pet1-Di* mice ($p < 0.04$) and greater locomotor activity in *Drd1a-Pet1-Di* mice compared with that in sibling controls on session 5 ($p < 0.01$), in follow-up to the genotype × session interaction ($F[4, 68] = 2.5$, $p < 0.047$).

Discussion

Dose-related effects of cocaine and CNO neutrality in the CPP assay

In both pilot experiments with *r2Hoxa2* and *Drd1a* genotypes, behaviorally active doses of cocaine generally included 10.0 and 17.8 mg/kg but not 1.0 and 3.0 mg/kg cocaine, as determined by horizontal locomotor activity during conditioning and cocaine preference after conditioning. These results are in line with wild-type C57BL/6 mice, whereby cocaine at doses ≥ 4.0 mg/kg enhanced unconditioned locomotor activity and produced significant CPP (George, 1989; Brabant et al., 2005; Orsini et al., 2005; Song et al., 2013). Across the *r2Hoxa2-Pet1-Di*, *Drd1a-Pet1-Di*, and respective sibling control mice used in the present study, 17.8 mg/kg cocaine produced a greater magnitude of locomotor activity during conditioning than 10.0 mg/kg cocaine. The consistency of this internal behavioral control measure suggests that CNO-Di-triggered neuronal inhibition in our transgenically engineered mice produced biologically meaningful outcomes.

Although neither we nor others have determined if CNO by itself produces conditioned place preference or aversion, it is unlikely to do so based on results from several studies involving reward-related testing as well as facets of the work presented herein. In one study (Guarino et al. 2020), CNO did not alter consummatory behavior compared with vehicle in normal Wistar rats during a reward devaluation task of sucrose consumption, suggesting that CNO is inherently neither rewarding nor aversive. In another study (Bock et al. 2013), CNO did not alter progressive ratio breakpoints for self-administered cocaine compared with vehicle in control mice, again suggesting that CNO is inherently neither rewarding nor aversive. Lastly, in our study, if CNO were to have aversive properties, then when injected repeatedly during conditioning in sibling control mice, preference for the cocaine-paired compartment in the CPP development test would have been reduced or eliminated. Instead, sibling control mice exhibited a preference for the cocaine compartment. It remains possible that CNO had rewarding properties in our assay. When injected repeatedly during 17.8 mg/kg cocaine conditioning (but not 10.0 mg/kg cocaine conditioning), the sibling control mice in the CPP development test exhibited a stronger preference for the cocaine compartment than in the CPP expression test in experiments 1 and 2, but the studies cited above argue against this interpretation.

CNO-Di-triggered neuronal inhibition alters cocaine memory in *r2Hoxa2-Pet1-Di* mice

In *r2Hoxa2-Pet1-Di* mice receiving behaviorally active cocaine doses during conditioning, acute CNO-triggered inhibition of *r2Hoxa2-Pet1* neurons during the post-conditioning period did not influence expression of cocaine CPP. Nor did repeated CNO administration during conditioning influence the initial development of cocaine CPP, but it did cause *r2Hoxa2-Pet1-Di* mice to maintain greater cocaine preference than sibling controls across the post-conditioning period after conditioning with 17.8 mg/kg cocaine. Thus, a more persistent cocaine memory developed and/or a greater resistance to extinction emerged with a high cocaine dose after repeatedly suppressing *r2Hoxa2-Pet1* neurons during conditioning. It was not surprising that group differences in extinction were detected only after conditioning with 17.8 mg/kg cocaine during CPP development testing in *r2Hoxa2-Pet1-Di* mice and their sibling controls. In previous studies, significant across-session extinction of cocaine CPP was associated with a relatively stronger preference for cocaine (Abraham et al. 2016; Bernardi and Lattal, 2012). In experiment 1, initial cocaine preference in sibling control mice was relatively stronger after conditioning with 17.8 mg/kg cocaine during CPP development testing (mean S^+ difference > 250 s) compared with conditioning with 17.8 mg/kg cocaine during CPP expression testing and to conditioning with 10.0 mg/kg cocaine under both testing conditions (mean S^+ differences < 200 s). Sibling control mice conditioned with 17.8 mg/kg cocaine + CNO during CPP development testing exhibited an expected steep decline in cocaine preference from post-conditioning session 2 to session 6. Extinction of cocaine preference followed a similar trajectory as these sibling controls in both *Drd1a-Pet1-Di* and sibling control mice during CPP development testing in experiment 2 as well. Consequently, the lack of significant changes in cocaine preference under these same conditions in *r2Hoxa2-Pet1-Di* mice may reflect an extinction deficit, resulting in between-group differences compared with sibling controls in the time course analysis. These findings suggest that *r2Hoxa2-Pet1* neurons normally may serve to limit the durability or strength of cocaine memory during abstinence and facilitate its extinction, but otherwise not impact the initial development or expression of cocaine CPP. This profile is consistent with the moderate to dense projections of *r2Hoxa2-Pet1* neurons to multiple sites underlying associative learning and memory, such as the medial prefrontal cortex, hippocampus, and basolateral amygdala, and the relatively weaker projections to reward-relevant sites, such as nucleus accumbens (Bang et al., 2012). Along these lines, chronic administration of 5-HT uptake inhibitors, which increase extracellular 5-HT within the prefrontal cortex, hippocampus, and amygdala (Bosker et al., 2001; Hervas et al., 2000), facilitates extinction learning and retention in mice (Karpova et al., 2011).

CNO-Di-triggered neuronal inhibition alters cocaine reward in *Drd1a-Pet1-Di* mice

In *Drd1a-Pet1-Di* mice receiving behaviorally active cocaine doses during conditioning, we observed that acute CNO administration on post-conditioning session 2 transiently reduced the heightened cocaine preference expressed in these mice prior to any CNO-Di-triggered neuronal inhibition. Cocaine preference returned to elevated levels in *Drd1a-Pet1-Di* mice by post-conditioning session 4, providing strong within-subject confirmation that this phenotype is expressed reliably in the absence of CNO-Di-triggered neuronal inhibition of *Drd1a-Pet1-Di* neurons during post-conditioning preference testing. During CPP development testing, the magnitude of cocaine preference was similar in *Drd1a-Pet1-Di* and sibling control mice; thus, it appears that repeatedly inhibiting *Drd1a-Pet1* neurons with CNO just prior to receiving a behaviorally active cocaine dose during conditioning can persistently block this basal phenotype in *Drd1a-Pet1-Di* mice.

It was surprising that triple transgenic *Drd1a-Pet1-Di* mice receiving behaviorally active doses of cocaine during conditioning exhibited heightened cocaine CPP as compared with sibling controls before neuronal inhibition with CNO. Typically, animals harboring triggerable Di receptors behave similarly to controls prior to any CNO administration, as demonstrated in experiment 1 with *r2Hoxa2-Pet1-Di* mice as well as in previous reports of mice expressing Di (Ray et al., 2011; Brust et al., 2014; Dosumu-Johnson et al., 2018; Teissier et al., 2015; Stachniak et al., 2014; Kerstetter et al., 2016). Subsequently, we sorted the sibling control mice by genotype resulting from *Drd1a-Pet1-Di* crosses to determine if enhanced cocaine reward also was expressed in a subset of sibling controls. No broad differences in the preference scores of single and double transgenic sibling control mice were observed (Table S1). A caveat, however, is that three subgroups of sibling control genotypes consisted of only 2–3 mice, making statistical comparisons impossible to perform. Nonetheless, these findings suggest that the manifestation of heightened cocaine CPP likely required the presence of all three transgenes (*RC::FPDi*, *Pet1::Flpe*, and *Drd1a::cre*) in addition to testing the transgenic mice under conditions whereby the targeted neurons were not inhibited before receiving behaviorally active cocaine doses during conditioning. Support for the possibility of a basal phenotype in certain cell types in the absence of triggering Di signaling with CNO was presented in a recent review on DREAAD technology (Roth, 2016). Although additional research is necessary to elucidate the mechanisms underlying this unanticipated phenotype, we speculate that prior to any CNO-Di-triggered neuronal inhibition, the intrinsic excitability of *Drd1a-Pet1-Di* neurons might be higher in transgenic mice exposed to behaviorally active doses of cocaine relative to transgenic mice without this particular transgene combination. Altered intrinsic properties of *Drd1a-Pet1* neurons might arise from a combination of factors: these cells may have a greater than normal sensitivity to expression of Di—perhaps through Di-sequestering of endogenous inhibitory G protein signaling components—thereby diminishing transduction of endogenous inhibitory tone by these cells and rendering them more excitable when exposed to cocaine. Such hyper-excitability might enhance cocaine CPP, which might then be ameliorated in the presence of CNO-Di-triggered inhibition, consistent with the findings reported here. Additionally, a proportion of *Drd1a-Pet1* cells express the dopamine type 2 receptor (DRD2) (Niederkofler et al., 2016), and independent studies in rodents demonstrated that DRD2 agonists can enhance CPP (Merritt and Bachtell, 2013) and other forms of cocaine-seeking behavior (Self et al., 1996; Fuchs et al., 2002)—perhaps aspects mediated through DRD2 signaling on *Drd1a-Pet1* neurons. It remains unclear, however, whether DRD2 signaling in these cells, canonically mediated through inhibitory G proteins, is crippled to a degree due to expression of the exogenous Di receptor, which may compete for Gi/o proteins. Evidence further suggests that cocaine can indirectly activate DRD2 (Anderson et al., 2006), thus perhaps also contributing to the suggested role of *Drd1a-Pet1* neurons in enhancing cocaine CPP.

Taking a different strategy, other investigators demonstrated that CNO-mediated activation of Dq (excitatory DREADD) specifically of the full population of dorsal raphe *Pet-1* neurons projecting to the nucleus accumbens abolishes cocaine CPP in mice (You et al., 2016). Our prior work showed that *Drd1a-Pet1* neurons send significant projections to the nucleus accumbens (Niederkofler et al., 2016). Therefore, *Drd1a-Pet1* neurons may have a specialized role in cocaine reward among the dorsal raphe *Pet-1* neurons, given that CNO-mediated activation of Di in this 5-HT *Pet-1* neuron subtype reduced the heightened expression of cocaine CPP in *Drd1a-Pet1-Di* mice. This functional difference between *Drd1a-Pet1* neurons and the full population of dorsal raphe *Pet1* neurons for expression of cocaine CPP supports the view that 5-HT-mediated function does not always conform to anatomically defined groupings per se but rather to developmental molecular expression, even when said neurons reside adjacently intermingled within the same anatomical nucleus, as

reported previously for serotonergic regulation of brainstem breathing dynamics (Brust et al., 2014).

CNO-Di-triggered neuronal inhibition and locomotor activity

Acute CNO injection during the post-conditioning session 2 during CPP expression testing reduced locomotor activity compared with saline injection in animals from both experiments regardless of genotype and cocaine dose employed during the conditioning phase. While suggestive of a non-specific motor effect of acute 10.0-mg/kg CNO administration, this is likely not the case based on several additional observations. First, within each genotype, there was less locomotor activity on session 2 comparted with that on session 1, whether mice received CNO on session 2 (CPP expression testing condition) or did not receive CNO on session 2 (CPP development testing condition). Secondly, the time course analysis during CPP expression testing demonstrated that locomotor activity was relatively stable across sessions 2–6 within each genotype (except for session 4), suggestive of a natural decline in locomotor activity from session 1 to sessions 2–6 in the cocaine-free state. This may be due to the relative novelty of having access to the entire CPP chamber on post-conditioning session 1, after having completed 8 daily conditioning sessions while confined to one or the other compartment. An examination of the three pre-conditioning preference test sessions during CPP expression testing supports the view that relative novelty of the chamber influences the magnitude of locomotor activity, as counts across pre-conditioning sessions 1–3 declined from 1204 ± 24 to 957 ± 25 and 945 ± 28 in sibling controls, from 1209 ± 33 to 932 ± 37 and 861 ± 35 in *Drd1a-Pet1-Di* mice, and from 1149 ± 33 to 879 ± 37 and 850 ± 41 in *r2Hoxa2-Pet1* mice. These findings are in general agreement with past studies showing that the silencing of *Drd1a-Pet1* and *r2Hoxa2-Pet1* neurons had no influence on horizontal locomotor activity (Teissier et al., 2015; Niederkofler et al., 2016). The differential effects of inhibiting *r2Hoxa2-Pet1* and *Drd1a-Pet1* neurons on cocaine-induced locomotor activity vs. cocaine reward and memory are not surprising. Locomotor stimulant actions of cocaine require activation of striatal circuits (Beeler et al., 2009), whereas the reward and memory aspects of cocaine rely on activation of mesolimbic circuits (Everitt et al., 2001). Inhibiting *r2Hoxa2-Pet1* and *Drd1a-Pet1* neurons likely would influence mesolimbic circuits more so than striatal circuits, as suggested by the moderate to dense projections to mesolimbic sites and much weaker projections to the dorsal striatum (Bang et al., 2012; Niederkofler et al., 2016).

Repeated CNO injections during CPP development testing modestly and temporarily reduced locomotor activity non-specifically but did not impact the ability of cocaine to increase locomotor activity relative to saline injection. However, specific effects of repeated CNO-mediated neuronal inhibition during conditioning emerged over the cocaine-free post-conditioning period, exhibiting as greater locomotor activity in *Drd1a-Pet1-Di* than sibling control mice conditioned with 17.8 but not 10.0 mg/kg cocaine and no change in locomotor activity in *r2Hoxa2-Pet1* compared with sibling control mice conditioned with 10.0 or 17.8 mg/kg cocaine. These findings are in line with past studies using the *tox* (light chain from tetanus toxin) silencing transgene to show that constitutively silenced *Pet1* neurons arising from the *En1*+ hindbrain territory of the isthmus and rhombomere 1 (a subset that includes *Drd1a-Pet1* neurons) were associated with hyperactivity (Kim et al., 2009). Constitutively silenced *Pet1* neurons arising from rhombomere 2 (*r2Hoxa2-Pet1* neurons) were neutral with respect to baseline locomotor activity (Okaty et al., 2015). The basis for the emergent effect of repeated silencing of *Drd1a-Pet1* neurons on locomotor activity during the cocaine-free post-conditioning is unclear at present, but their silencing might reflect a stabilizing influence on cocaine-conditioned locomotor responses conventionally formed through Pavlovian association (Johnson et al., 2012).

Conclusions

Taken together with previous research (Kim, 2009; Okaty, 2015; Brust, 2014; Niederkofler et al., 2016; Tessier et al., 2017; Hennessy et al., 2017; Ren et al., 2018; Ren et al., 2019), genetic and developmental differences may underlie functional differences across the broader serotonergic neuron population. Differentiating *Pet1* 5-HT neurons by more than just their anatomy may help define 5-HT neuron subtypes with specific functions relating to distinct diseases. Using a *Pet1* neuron subtype–specific chemogenetic approach, novel insights were gleaned concerning the role of 5-HT neurons in cocaine action—insights that would likely otherwise be missed if more global manipulations were used. *r2Hoxa2-Pet1* neurons might contribute to lessening the durability or strength of cocaine memory during abstinence, whereas *Drd1a-Pet1* neurons might contribute to the magnitude of cocaine reward. It is possible that molecular genetic differences in the intrinsic properties of *r2Hoxa2-Pet1* and *Drd1a-Pet1* neurons are mechanistic features that factor into the well-known individual differences in vulnerability to cocaine addiction (George and Koob, 2010). Along these lines, early postnatal development is thought to be a particularly sensitive period during which outside stressors can influence maturation of 5-HT neuron excitability in the dorsal raphe, leading to 5-HT system dysregulation and pathological emotional states (Rood et al., 2014).

Acknowledgments The authors thank Emery Mokler, Carly Rose Willing, and Morgan Freret for the technical assistance.

Funding information The National Institutes of Health grant DA036056 funded this research.

Compliance with ethical standards

All housing and behavioral procedures were approved by the respective Institutional Animal Care and Use Committee (IACUC) at Harvard Medical School and Boston University.

Conflict of interest The authors declare that they have no competing interests.

Ethical approval All animal housing, generation, re-derivation, breeding, and behavioral procedures were approved by the respective Institutional Animal Care and Use Committee (IACUC) at Harvard Medical School and Boston University.

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