Plasticity in neuronal circuits enables animals to adapt to an ever-changing environment. However, the loci and nature of experience-dependent changes in circuit function that drive adaptive and pathological behaviours remain largely unknown. Modern techniques such as optogenetics and chemogenetics permit the sequential screening of targeted elements in circuits with complex input and output patterns, but unbiased approaches are necessary to identify new components that play critical roles in the behavioural changes of interest. One such approach uses the expression of immediate early genes to identify the neuronal ensembles activated by a defined experience, but, as currently used, this approach does not reveal the connectivity of these ensembles. Brain imaging techniques such as functional MRI or positron-emission tomography also provide insights into unexpected experience-dependent macroscopic activity changes, but lack cellular resolution. Thus, there is a need for additional methods that facilitate unbiased identification of the circuit substrates of experience-dependent behavioural changes. Here, we present evidence that rabies virus-based monosynaptic tracing facilitates screening of circuit elements that contribute to behavioural changes by allowing whole-brain mapping of monosynaptic inputs to defined starter neuronal populations and their input–output relationships.

To test the utility of a rabies virus-based approach for screening of behaviourally relevant experience-dependent circuit adaptations, we initially focused on the ventral tegmental area (VTA), a circuit node that is critical for a variety of experience-dependent behaviours. The diverse array of inputs to and output relationships of dopamine and GABA (γ-aminobutyric acid) cells in the VTA have been extensively elucidated using rabies virus methods. We used drugs of abuse as a robust trigger of experience-dependent plasticity, focusing on cocaine, which elicits long-lasting behavioural adaptations including locomotor sensitization (LMS) and conditioned place preference (CPP). Our unbiased input screen revealed an unexpected critical role for the GPe, which has been implicated in motor control, habit formation and Parkinson’s disease but not studied in relation to addiction-related behaviours triggered by drugs of abuse. Our findings provide proof of principle for the utility of this approach while implicating the GPe in the establishment of drug-evoked behavioural plasticity.

Screen for cocaine–induced input changes
Cocaine administration induces modification of synapses on dopamine and GABA neurons in the VTA (VTA-DA and VTA-GABA neurons, respectively) but the identities of cells providing those inputs are largely unknown. To test whether rabies virus monosynaptic tracing might reveal the identities of inputs altered by cocaine, we first used the rabies monosynaptic input tracing technique where animals were given a single injection of cocaine (15 mg kg⁻¹) or saline one day before injection of the rabies virus, RVG (Fig. 1a). Unbiased analysis of the labelled input cells from 22 brain regions comprising more than 90% of long-range inputs to VTA-DA neurons revealed that although the global maps were quantitatively similar, labelling proportionally increased or decreased in cocaine-treated animals in a small subset of regions, with inputs from the GPe displaying the largest proportional change (Fig. 1b).

To determine whether similar labelling changes occurred in response to different classes of abused substances, as occurs with synaptic adaptations onto VTA-DA neurons, we administered single doses of amphetamine, morphine, nicotine, or the psychoactive but non-addictive substance fluoxetine. The drugs of abuse increased labelling of GPe cells whereas fluoxetine did not (Fig. 1c, Extended Data Fig. 1a). As previous work using rabies virus revealed that VTA-DA and VTA-GABA neurons receive similar inputs, we next used the GAD2Cre mouse driver line to test whether cocaine induced similar changes in labelling of cells making monosynaptic contacts onto midbrain GABA neurons in the VTA and the nearby substantia nigra pars reticulata (SNr) (Fig. 1d). Cocaine again elicited a proportional increase in labelling of GPe cells compared to saline injections (Fig. 1e).
**Cocaine enhances GPe–PV neuron activity**

The four brain regions with the largest drug-induced change in labelling were the GPe, anterior cortex (Ant. Ctx.), medial habenula (MHB), and nucleus accumbens medial shell (NAcMedS) (Fig. 1c, Extended Data Fig. 1a). While the latter three have been previously implicated in the behavioural adaptations caused by drugs of abuse\(^1\), the potential role of the GPe has not been investigated, to our knowledge. To investigate what the increase in rabies virus labelling of GPe inputs onto VTA-DA neurons may tell us about the effects of cocaine, we used parvalbumin (Pvalb)-Cre or Pvalb-Flp mouse driver lines to specifically target parvalbumin neurons in the GPe (GPe-PV cells) (as the majority of rabies-labelled GPe cells making synapses onto VTA-DA neurons co-stained with parvalbumin (305 of 367; Extended Data Fig. 1b)). Visualization of axons of GPe-PV neurons expressing a membrane-tagged GFP demonstrated that these cells send major projections to a range of brain regions, including the forebrain and thalamus (Extended Data Fig. 2a, b). Consistent with previous work\(^1\), the main GPe projection to the midbrain was to the SNr, which contains mostly GABA neurons\(^2\), while dopamine neuron-containing nuclei such as the substantia nigra pars compacta (SNc) or VTA received fewer inputs (Extended Data Fig. 2c, d).

To determine whether the cocaine-induced increase in rabies virus labelling of GPe cells reflected an increase in the number of synaptic contacts, we injected the adeno-associated virus (AAV) AAV-FLEX\(^{dmg-P}_{-}\)-mGFP-2A-synaptophysin-mRuby, which labels putative presynaptic sites (Fig. 2a), into the GPe of Pvalb-Cre mice and imaged them after a cocaine or saline injection about 1 month later (Fig. 2b). The density and volume of labelled puncta in the SNr were indistinguishable in saline versus cocaine-treated animals (Fig. 2c, d), suggesting that there was no change in synapse number. Next, we assessed potential changes in synapse strength, as single doses of drugs of abuse can enhance the synaptic strength of both excitatory and inhibitory inputs into the ventral midbrain\(^1\). We injected a Flp-dependent AAV expressing channelrhodopsin (AAV-FLEX\(^{FRT-ChR2}\)) into the GPe of Pvalb-Cre mice and conducted whole-cell recordings from SNr neurons in acute slices about 1 month later. Calcium in the extracellular recording solution was replaced by strontium to enable measurement of quantal events\(^2\) (Fig. 2e, f). No change in the amplitude or frequency of quantal events was observed in slices prepared from cocaine- versus saline-treated mice (Fig. 2g, h), suggesting that cocaine did not elicit a change in the strength of the inhibitory synapses made by GPe-PE neurons onto SNr neurons.

Finally, we assessed whether the spontaneous activity of GPe-PV neurons in vivo was altered by cocaine by expressing the fluorescent Ca\(^{2+}\) indicator GCaMP6f in GPe-PV neurons and collecting population Ca\(^{2+}\)-dependent signals using fibre photometry (Fig. 2i). Although
cocaine administration had no significant acute effect, population activity had increased twofold by the following day (Fig. 2j, k). To assess whether this increase might reflect changes in intrinsic excitability, we injected AAV-FLEXloxP-GFP into the GPe of Pvalb-Cre mice and made targeted whole-cell current clamp recordings from acute GPe slices prepared one day after the mice received saline or cocaine injections (Fig. 2i). GPe-PV neurons from cocaine-treated mice were more excitable, in that more action potentials were generated by depolarizing current injection steps (Fig. 2m, n) with no change in the input resistances of the cells (Fig. 2n). These results demonstrate that a single exposure to cocaine triggers increases in the spontaneous activity and excitability of GPe-PV neurons.

**Input activity modulates rabies labelling**

To investigate whether the cocaine-induced changes in rabies virus labelling of GPe-PV input neurons result from the observed changes in their activity, we expressed transgenes in GPe-PV neurons that permit manipulation of their activity levels while simultaneously performing rabies virus transsynaptic labelling. Chronic activity manipulations bidirectionally influenced the extent of GPe neuronal labelling: chronic inhibition of GPe-PV neuron activity with the inhibitory DREADD hM4Di, Kir2.1 or the tetanus toxin light chain (TeTxLc), which inhibits presynaptic vesicle release 22—prevented cocaine-induced LMS (Fig. 4a–c), while having no effect on basal locomotion and only a modest effect on cocaine-induced locomotion upon initial administration (Extended Data Fig. 3). Furthermore, we performed the same genetic manipulations on D1 receptor-expressing medium spiny neurons (MSNs) in the NAcMedS, the labelling of which was decreased by drugs of abuse (Fig. 1c, Extended Data Fig. 1a, c). Parallel to the effects of manipulating GPe-PV neurons, chronic NAcMedS inhibition using hM4Di or K\textsubscript{\textalpha}2.1 reduced labelled inputs into the NAcMedS, whereas activation with hM3Dq enhanced NAcMedS labelling (Fig. 3h). Unexpectedly, although NAcMedS inhibition did not alter labelling of GPe neurons, NAcMedS activation increased labelled GPe inputs (Fig. 3g), suggesting that activation of D1 MSNs in the NAcMedS indirectly increases GPe activity. Together, these experiments provide evidence that rabies-mediated transsynaptic labelling from starter neurons is influenced by the level of activity in input cell populations. Furthermore, they support the hypothesis that cocaine and other drugs of abuse chronically increase the level of GPe-PV neuron activity and that this accounts for the observed increase in GPe neuron labelling by the rabies virus.

**Behavioural changes require GPe activity**

To determine whether the cocaine-elicted changes in GPe-PV neuron activity revealed by rabies virus tracing are functionally relevant and necessary for cocaine-induced behavioural adaptations, we tested whether inhibition of GPe-PV neurons influenced two forms of cocaine-induced behavioural plasticity, LMS and CPP. Inhibition of activity in GPe-PV neurons using expression of any one of three transgenes—encoding hM4Di, K\textsubscript{\textalpha}2.1 or the tetanus toxin light chain (TeTxLc), which inhibits presynaptic vesicle release 23—prevented cocaine-induced LMS (Fig. 4a–c), while having no effect on basal locomotion and only a modest effect on cocaine-induced locomotion upon initial administration (Extended Data Fig. 3). Furthermore,
Figure 3 | Bidirectional modulation of rabies labelling by activity manipulations. a, A mixture of AAV-FLEXloxP-TC and AAV-FLEXloxPG was injected into the VTA while an AAV expressing a Flp-dependent YFP, hM4Di, K$_{2.1}$ or hM3Dq was injected into the GPe. b, Rabies labelling in GPe (scale bar, 1 mm). c–e, GPe manipulation. f, Experimental strategy. g, Quantification of labelled inputs in GPe. g-axes show labelled GPe inputs (NAcLatS + NAcCore inputs). Combined controls (uninjected and YFP) were assigned a value of 1. gPe inhibition reduced (hM4Di, $P = 0.004$; K$_{2.1}$, $P < 0.0001$) and activation increased (hM3Dq, $P = 0.016$) labelled inputs. h, Quantification of labelled inputs in NAcMedS (hM4Di, $P = 0.98$; K$_{2.1}$, $P = 0.90$; hM3Dq, $P = 0.88$). i, NAcMedS manipulation. j, Experimental strategy. k, NAcMedS-D1 inhibition had no effect on labelling of GPe inputs (hM4Di, $P = 0.13$; K$_{2.1}$, $P = 0.65$) while activation increased labelling (hM3Dq, $P < 0.001$). l, NAcMedS-D1 inhibition decreased (hM4Di, $P = 0.02$; K$_{2.1}$, $P = 0.03$) while activation increased (hM3Dq, $P = 0.007$) labelling of NAcMedS inputs. The schematics of the mouse brain in this figure were adapted from ref. 33.

there was a positive correlation between the number of rabies-labelled GPe inputs and the magnitude of LMS in a two-day injection protocol for cocaine as well as other addictive drugs (Extended Data Fig. 4).

Similar results were obtained for cocaine-elicited CPP: using a four-day protocol (Fig. 4d), inhibition of GPe-PV activity through expression of hM4Di, K$_{2.1}$ or TeTxLc prevented CPP (Fig. 4e, f). Inhibition of GPe-PV neurons with hM4Di also prevented morphine-induced LMS and CPP (Extended Data Fig. 5), implying that the role of these neurons may generalize to other abused substances.

A limitation of these experiments is that GPe-PV neurons project to several brain regions (Extended Data Fig. 2) and thus the transgene-mediated inhibition of these neurons was not target-specific. To limit the expression of an inhibitory transgene specifically to those GPe-PV neurons that project to the midbrain, we injected the retrogradely transported CAV-FLEXloxP-Flp into the midbrain of Pvalb-Cre mice and the Flp-dependent AAV-FLEXPG-K$_{2.1}$ into the GPe, enabling K$_{2.1}$ expression to be restricted to Cre-expressing GPe-PV neurons projecting to the midbrain (Extended Data Fig. 6a). This manipulation prevented both cocaine-elicited LMS and CPP (Extended Data Fig. 6b, c). However, subsequent axon tracing experiments revealed that the GPe-PV neurons projecting to the midbrain collateralize extensively (Extended Data Fig. 7), limiting the interpretation of this experiment. To inhibit the GPe-PV neuron projections within the midbrain specifically, we expressed hM4Di in GPe-PV neurons and infused slow-release clozapine N-oxide (CNO) microspheres into the midbrain (Fig. 4g). This local inhibition was sufficient to block cortex-elicited LMS (Fig. 4h) and CPP (Fig. 4i). After waiting a week to allow depletion of CNO, the same mice were re-tested using the same protocols, and expressed significant LMS and CPP (Fig. 4h, i). These results demonstrate that activity in the projection of GPe-PV neurons to the midbrain is necessary for two forms of cocaine-induced behavioural plasticity.

GPe-PV neurons disinhibit VTA–DA neurons

Does the cocaine-induced increase in activity in inhibitory GPe-PV neurons influence dopamine neuron activity, which is required for induction of LMS and CPP (Extended Data Fig. 8)? Given that the major projection from GPe-PV neurons to the midbrain is into the SNr (Extended Data Fig. 2), a simple hypothesis is that GPe-PV neurons strongly inhibit SNr neurons that tonically inhibit dopamine neurons. Thus, activation of GPe-PV neurons would increase dopamine neuron activity for a single mouse. c, GPe-PV neuron inhibition blocked cocaine-induced LMS (hM4Di, $P = 0.0002$; K$_{2.1}$, $P = 0.007$; TeTxLc, $P = 0.008$). d–f, Conditioned place preference. g, Procedure to test CPP during GPe-PV neuron inhibition. h, Trace from YFP mouse during post-test.

f, GPe-PV neuron inhibition prevented CPP (hM4Di, $P = 0.019$; K$_{2.1}$, $P = 0.030$; TeTxLc, $P = 0.029$). g–i, Inhibition of GPe-PV projections to midbrain. g, Slow-release CNO microspheres were injected into ventral midbrain in animals expressing hM4Di or YFP in GPe-PV neurons. h, i, Both LMS (h; $P = 0.0005$) and CPP (i; $P = 0.0094$) were blocked in hM4Di-expressing animals. When tested again after CNO washout, the same mice developed LMS (h; $P = 0.047$) and CPP (i; $P = 0.0078$). The schematics of the mouse brain in this figure were adapted from ref. 33.

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activity via disinhibition, despite some direct synaptic inhibition of dopamine neurons. We tested this hypothesis in several ways. First, to examine the functional strength of GPe-PV neuron connections to midbrain dopamine and GABA neurons, we injected AAV-FLExloxP-ChR2 into the GPe of either DAT-Cre;Pvalb-Flp (DAT is also known as Slc6a3) or Gad2-Cre;Pvalb-Flp double transgenic mice, AAV-FLEXwIP2-GFP into the VTA to label dopamine or GABA neurons (Fig. 5a), and retrobeads into either the NAcMed or nucleus accumbens lateral shell (NAcLatS) to demarcate one of two VTA-DA subpopulations whose inputs are modified by cocaine. We then measured monosynaptic connectivity GPe–midbrain. a–c, Monosynaptic connectivity between GPe and midbrain. a, AAV-FLExloxP-ChR2 was injected into the GPe, AAV-FLExlop2-GFP injected into the VTA, and retrobeads injected into the NAcLatS or NAcMedS of DAT-Cre;Pvalb-Flp or Gad2-Cre;Pvalb-Flp mice. Whole-cell recordings were made from identified midbrain neurons in acute slices. b, Example light-evoked IPSCs. c, Quantification of per cent connectivity and IPSC amplitude for each cell type. d–f, Local inhibitory inputs to VTA-DA neurons. d, AAV-FLEXloxP-TC66T and AAV-FLEXlop2-GFP were injected into the VTA of DAT-Cre mice, followed two weeks later by BVAIG. e, Sample labelling of midbrain section (scale bar, 1 mm). f, Quantification of labelled local inhibitory inputs. g–i, GPe control of dopamine neuron activity. g, Cre-dependent AAV expressing YFP, hM4Di, K2.1, or ChR2 were injected into the GPe of Pvalb-Cre mice followed by quantification of Fos labelling after manipulations. h, i, Sections of ventral midbrain showing Fos labelling (green) and tyrosine hydroxylase (TH) labelling (magenta) in animals receiving cocaine injections and expressing YFP (h) or hM4Di (i) in GPe. Arrows indicate Fos+ neurons co-expressing tyrosine hydroxylase (scale bar, 200 μm). j, Quantification of activated dopamine neurons (Fos+ TH+) relative to all activated ventral midbrain neurons (Fos+; YFP, P=0.029; ChR2, P<0.0001). k–m, GPe inhibition of dopamine neurons. k, Flp-dependent bReaChES was injected into the GPe and a Cre-dependent GCaMP6f was injected into the VTA of DAT-Cre;Pvalb-Flp mice. Fibre photometry traces during consecutive 10-min epochs. m, VTA-DA neurons were more active during light-on than light-off (one-way ANOVA, P=0.014; post hoc tests 0–10 vs. 10–20 min, P=0.008; 10–20 vs. 20–30 min, P=0.039). n, Proposed circuit diagram before and after cocaine. Sizes of cell bodies and arrows represent activity strength. The schematics of the mouse brain in this figure were adapted from ref. 33.

Second, we tested whether manipulating activity in SNr-GABA neurons could alter the development of LMS and CPP. Activation of SNr-GABA neurons with hM3Dq prevented the development of LMS and CPP (Extended Data Fig. 10a–c), whereas concurrently inhibiting SNr-GABA neurons with hM4Di during chronic GPe-PV neuron inhibition rescued cocaine-mediated behavioural plasticity (Extended Data Fig. 10d–f). These results suggest that GPe-PV neuron inhibition prevents LMS and CPP by enhancing the activity of SNr-GABA neurons.

Third, we examined whether SNr-GABA neurons provide direct inhibition to dopamine neurons using a modified rabies virus input tracing strategy25 to label local inputs onto dopamine neurons and combining this with in situ hybridization for Gad1 and Gad2 mRNA (Fig. 5d, e). Quantitatively, SNr-GABA neurons were the second-largest source of inhibitory inputs to dopamine neurons (Fig. 5f).

Fourth, we tested whether changes in GPe-PV neuron activity influenced integrated VTA-DA neuron activity using Fos labelling. In YFP-expressing mice, acute cocaine administration caused a clear increase in Fos+ dopamine neurons compared to saline-injected mice 90 min after injection (Fig. 5j). Inhibition of GPe-PV neurons with hM4Di had no effect in saline-treated animals but completely prevented the increase in cocaine-treated mice, as did expression of Kir2.1 (Fig. 5j). Conversely, activation of GPe-PV neurons by ChR2 increased Fos expression in dopamine neurons to a degree similar to that elicited by cocaine (Fig. 5j).

Finally, to test the effect of activating GPe-PV neurons on VTA-DA neuron activity directly in vivo, we injected a Flp-dependent red-shifted opsins (bReaChES)26 into the GPe and a Cre-dependent GCaMP6f into the VTA of DAT-Cre;Pvalb-Flp double transgenic mice, and recorded population activity from midbrain dopamine neurons with fibre photometry while optically activating GPe-PV neurons (Fig. 5k). Stimulation of GPe-PV neurons caused a small but significant increase in GCaMP6f fluorescence in VTA-DA neurons (Fig. 5l, m). Together, these results indicate that GPe-PV neurons control VTA-DA neuron activity through disynaptic disinhibition via SNr-GABA neurons (Fig. 5n).
Concluding remarks

The association of specific experience-dependent circuit changes with corresponding behavioural adaptations has been hampered by a lack of suitable methodology. While most investigations of circuit function rely on a candidate approach that takes advantage of cell-type specific manipulations, such studies are limited by the requirement for a priori assumptions regarding the roles of targeted populations. Given the complexity of circuit mechanisms that mediate animal behaviours, unbiased approaches offer advantages for identifying the array of specific neuroplastic changes that drive behavioural changes. Here, we demonstrate that neural activity influences the extent of labelling by the rabies monosynaptic input tracing method. This property enabled us to identify circuit elements that were modified by experience in a behaviourally relevant manner. Specifically, the activity-dependence of rabies labelling enabled us to identify an underappreciated circuit node, the GPi, as a key player in cocaine-induced behavioural adaptations. As a core component of the dorsal striatal indirect pathway implicated in motor control and associated disorders, the GPi had not previously (to our knowledge) been directly linked to addictive behaviours. Using a number of different assays, we demonstrated that the effect of enhanced GPi input to the midbrain is to increase VTA-DA neuron activity via a mechanism of disinhibition (Fig. 5n). Our findings provide a novel circuit mechanism for influencing VTA-DA neuron activity, and confirm that this mechanism has a critical role in drug-induced behavioural adaptations.11,28 (Extended Data Fig. 8). Given that the rabies virus transsynaptic technique has been used successfully to map inputs onto cell populations in many brain areas,5–9,29–32, we anticipate that this method will be generalizable and complementary to other circuit analysis methods1,2 for elucidating how activity changes in connected ensembles orchestrate features of complex circuits.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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METHODS

No statistical methods were used to predetermine sample size. Experiments described in Figs. 1a, 1b, –1, –3, and 5g–j and Extended Data Figs 1a, 4, 5, 6, 8 and 10 were randomized and investigators were blinded to allocation and outcome assessments; all other experiments were not randomized and investigators were not blinded.

Mice and viral procedures. Generation and characterization of the DAT-Cre, GAD2-Cre, Pvalb-Cre, Pvalb-Floxed, A2a-Cre, and D1-tdTomato mouse lines have been described. Mice were housed on a 12-hour–12-hour dark cycle with food and water ad libitum. Males and females from a mixed CD1 and C57BL6 background were used for all experiments in approximately equal proportions. All surgeries were done under isoflurane anesthesia. All procedures complied with the animal care standards set forth by the National Institute of Health and were approved by Stanford University’s Administrative Panel on Laboratory Animal Care and Administrative Panel of Biosafety.

Construction of viruses containing \( hM3Dq \)-mCherry, \( hM4Di \)-mCherry, \( mGFP \), and \( eGFP \) were constructed as reported previously. For construction of viruses containing YFP, \( K_p, 2.1 \)–2A-GFP, \( hM4Di \)-mCherry, and \( hM3Dq \)-mCherry, \( pAAV-hsyn1-FLExloxP-mGFP \), 2A-synaptophysin-mRuby, and \( pAAV-FLExloxP-YFP \) were used as templates. All inserts were cloned into the Ascl and Sall sites between the loxP or FRT sites. \( hM3Dq \)-mCherry or \( hM4Di \)-mCherry were PCR-amplified from \( pAAV-hsyn1-DIO-hM3Dq-mCherry \) or \( pAAV-hsyn1-DIO-hM4Di-mCherry \), respectively, and \( K_p, 2.1 \)–2A-GFP was modified from Xue et al. The CMV-FLEx-luxP-GFP-2A-TeXluc, AAV-CMV-FLEx-loxP-ChR2(H134R)-eYFP, AAV-hsyn1-FLExloxP-eYFP, and AAV-FLExloxP-GcMAP6f were purchased from the Stanford Gene and Viral Vector Core.

Transsynaptic tracing. Synaptic puncta quantification and FRAP studies were carried out as previously described, with minor modifications. For rabies tracing, 100 nl of a 1:1 volume mixture of \( AAV-CAG-FLExloxP-G \) and \( AAV-CAG-FLExloxP-G \) was injected into the VTA7, or whether a drug of abuse had been injected (Fig. 1, Extended Data Fig. 1a).

Rabies input tracing and targeted input activity manipulations. To trace inputs from VTA-DA neurons while manipulating input regions, we used either DAT-Cre, Pvalb-Floxed double heterozygotes (for GPe manipulations) or D1-tdTomato mice (for NAcMedS manipulations) between 4 and 6 weeks of age. To manipulate GPe-PV neurons, in DAT-Cre:Pvalb-Fip mice, 100 nl of a 1:1 volume mixture of \( AAV-CAG-FLExloxP-G \) and \( AAV-CAG-FLExloxP-G \) was injected into the VTA of both hemispheres, and 300 nl of each \( AAV-CMV-FLExloxP-hMADi \), \( AAV-CMV-FLExloxP-hMDq \), \( AAV-CMV-FLExloxP-Kir2.1-2A-GFP \), \( AAV-CMV-FLExloxP-mCherry \), \( AAV-CMV-FLExloxP-mRuby \), \( AAV-CMV-FLExloxP-eGFP \), and \( AAV-FLExloxP-GcMAP6f \) were injected into the GPe. Both hemispheres were used for each mouse, and counted independently. Inputs from the NAcMedS were used for normalization, and \( AAV-CMV-FLExloxP-hMDq \) and \( AAV-CMV-FLExloxP-hMDq \) were injected into the same mice, as were \( AAV-CMV-FLExloxP-G \) and \( AAV-CMV-FLExloxP-G \). One month later, RVG was injected bilaterally into the VTA. To activate DREADDs, animals were injected once every 12 h with CNO for five days beginning immediately after RVG injection.

To manipulate NAcMedS-D1 neurons, in DAT-Cre:A2a-Cre mice, 100 nl of a 1:1 volume mixture of \( AAV-CMV-FLExloxP-G \) and \( AAV-CMV-FLExloxP-G \) was injected into the VTA. After recovery, mice were housed in a BSL2 facility for 5 days to allow virus spread and GFP expression. Values presented in Fig. 1 were not corrected for multiple comparisons.

For identification of local GABAergic inputs to VTA-DA neurons, 100 nl of a 1:1 volume mixture of \( AAV-CAG-FLExloxP-G \) and \( AAV-CAG-FLExloxP-G \) was injected into the VTA of 4–6-week-old mice. Thirteen days later, a single dose of cocaine (15 mg/kg), amphetamine (10 mg/kg), nicotine (0.5 mg/kg), morphine (10 mg/kg), fluoxetine (10 mg/kg) or saline was injected intraaperitonealy (IP), and the mouse was placed in a new cage for thirty minutes before being returned to its home cage. On the following day, RVG was injected into the VTA. After recovery, mice were housed in a BSL2 facility for 5 days to allow virus spread and GFP expression. Values presented in Fig. 1 were not corrected for multiple comparisons.

Quantification of Fos immunostaining. The number of Fos-positive cells was counted independently.

Inputs from the NAcCore and NAcMedS were used for normalization because the percentage of total rabies-labelled inputs from these regions was relatively invariant, regardless of whether injections were located more medially or laterally in the VTA7, or whether a drug of abuse had been injected (Fig. 1, Extended Data Fig. 1a).

Synaptic puncta quantification. Methods for synaptic puncta quantification were adapted from Mosca and Luo42 for use in mice. To quantify the volume and density of mRuby-labelled puncta from GFP–PV neuron termini in the SNr, 300 nl of \( AAV-CMV-FLExloxP-G \) were injected into the VTA. To examine the relationship between rabies-labelled inputs in the GPe and locomotor sensitization to cocaine injections using a two-dose procedure, in DAT-Cre mice, 100 nl of a 1:1 volume mixture of \( AAV-CMV-FLExloxP-G \) and \( AAV-CMV-FLExloxP-G \) was injected into the VTA on day 1. Mice were injected IP with saline, nicotine, or morphine, and placed in the open field chamber equipped with infrared lasers for motion tracking. On day 3, mice were injected with one of the following: saline (15 mg/kg), amphetamine (1 mg/kg), nicotine (0.5 mg/kg) or morphine (10 mg/kg), and placed in the same open field chamber. On day 4, RVG was injected into the VTA. On day 9, mice were injected with the same dose of the same drug they had received on day 3, and their locomotion was tracked in the open field. Experiments were terminated immediately thereafter. The rabies-labelled inputs into the GPe were quantified and normalized to the sum of inputs from the NACMedS and NACCore.

Synaptic projection quantification. Images were obtained and quantified as previously described, except that three sections were used for each region and were averaged to obtain the final value. Four brains were used for each experiment.

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brains sectioned on a vibratome in PBS. Sections were blocked with 10% normal donkey serum (NDS) and 2% BSA/0.5% triton-X100 in PBS (PBST), then stained with TH and Fos antibodies in 1% NDS in PBST at 4°C for four nights. Sections were rinsed three times for 30 min each at room temperature, followed by secondary antibody stain in 1% NDS in PBST at 4°C for two nights. Sections were then rinsed three times for 30 min each, and mounted on slides. The midbrain from three representative VTA-containing sections were then imaged on a Zeiss LSM 780 confocal microscope at 20× magnification, and the results averaged together for each brain. For activation of GPe neurons for Fos immunostaining, 300 nl of AAV-FLEXloxP-ChR2 was injected bilaterally into the GPe of Pvalb-Cre mice, and 200 μm, 0.39NA optical fibres were implanted over the VTA. For stimulation, the optical fibre was connected to a 473 nm laser diode (OEM Laser systems) through an FC/PC adaptor. Laser output was controlled using a Master-8 pulse stimulation, which delivered 5 ms light pulses at 20 Hz. Light output through the optical fibres was adjusted to 20 mW using a digital power meter console. Images were taken with a constant exposure time. Cells with a visually identifiable nuclear label by eye were considered Fos+.

**Immunohistochemistry.** The following primary antibodies were used: rat anti-mCherry, Life Sciences, 1:2,000; chicken anti-GFP, Aves Labs, 1:1,000; rabbit anti-TH, Millipore, 1:1,000; goat anti-Fos, Santa Cruz, 1:500. The following secondary antibodies were used from Jackson ImmunoResearch at a concentration of 1:250: donkey anti-chicken AlexaFluor488; donkey anti-goat AlexaFluor488; donkey anti-rat AlexaFluor555; donkey anti-goat AlexaFluor594; donkey anti-rabbit AlexaFluor647.

**Whole-cell recordings.** To assess coeruleine-induced changes in neuronal activity in vivo, fibre photometry was used as described previously. We injected 750 nl of AAV-FLEXloxP-GCaMP6f into the GPe of Pvalb-Cre mice, and implanted a 400μm diameter, 0.48NA optical fibre (Doric Lenses) at the same location. To test the effect of GPe-PV neuron stimulation on VTA-DA neuron activity, 300 nl of AAV-FLEXloxP-IBHreachES was injected into the GPe, and 750 nl of AAV-FLEXloxP-GCaMP6f was injected into the VTA of DAT-Cre-Pvalb-Flp mice. A 400 μm diameter, 0.48NA optical fibre was implanted over the VTA, and a 200 μm, 0.39NA fibre (made in-house) was implanted over the GPe. Implants were sealed to the skull with metal screws (Antrim Miniature Specialists), Metabond (Parkell), and Geristore dental epoxy (DenMat). Mice were allowed to recover for at least 4 weeks before experiments.

**Fibre photometry.** To observe the retrograde labelling with 1:250: donkey anti-chicken AlexaFluor488; donkey anti-goat AlexaFluor555; donkey anti-rabbit AlexaFluor647. For each brain, we calculated the percentage of time that the photometry trace was across the entire photometry trace during a given session. We conservatively estimated the area under the curve for each session, and then determined the percentage of that area that was above a threshold. To assess neural activity, the median absolute deviation (MAD) was calculated across the entire photometry trace during a given session. We conservatively estimated the area under the curve for each session, and then determined the percentage of that area that was above a threshold. To assess neural activity, the median absolute deviation (MAD) was calculated across the entire photometry trace during a given session. We conservatively estimated the area under the curve for each session, and then determined the percentage of that area that was above a threshold. To assess neural activity, the median absolute deviation (MAD) was calculated across the entire photometry trace during a given session. We conservatively estimated the area under the curve for each session, and then determined the percentage of that area that was above a threshold. To assess neural activity, the median absolute deviation (MAD) was calculated across the entire photometry trace during a given session. We conservatively estimated the area under the curve for each session, and then determined the percentage of that area that was above a threshold. To assess neural activity, the median absolute deviation (MAD) was calculated across the entire photometry trace during a given session. We conservatively estimated the area under the curve for each session, and then determined the percentage of that area that was above a threshold. To assess neural activity, the median absolute deviation (MAD) was calculated across the entire photometry trace during a given session. We conservatively estimated the area under the curve for each session, and then determined the percentage of that area that was above a threshold. To assess neural activity, the median absolute deviation (MAD) was calculated across the entire photometry trace during a given session. We conservatively estimated the area under the curve for each session, and then determined the percentage of that area that was above a threshold. To assess neural activity, the median absolute deviation (MAD) was calculated across the entire photometry trace during a given session. We conservatively estimated the area under the curve for each session, and then determined the percentage of that area that was above a threshold. To assess neural activity, the median absolute deviation (MAD) was calculated across the entire photometry trace during a given session. We conservatively estimated the area under the curve for each session, and then determined the percentage of that area that was above a threshold. To assess neural activity, the median absolute deviation (MAD) was calculated across the entire photometry trace during a given session. We conservatively estimated the area under the curve for each session, and then determined the percentage of that area that was above a threshold. To assess neural activity, the median absolute deviation (MAD) was calculated across the entire photometry trace during a given session. We conservatively estimated the area under the curve for each session, and then determined the percentage of that area that was above a threshold. To assess neural activity, the median absolute deviation (MAD) was calculated across the entire photometry trace during a given session. We conservatively estimated the area under the curve for each session, and then determined the percentage of that area that was above a threshold. To assess neural activity, the median absolute deviation (MAD) was calculated across the entire photometry trace during a given session. We conservatively estimated the area under the curve for each session, and then determined the percentage of that area that was above a threshold. To assess neural activity, the median absolute deviation (MAD) was calculated across the entire photometry trace during a given session. We conservatively estimated the area under the curve for each session, and then determined the percentage of that area that was above a threshold. To assess neural activity, the median absolute deviation (MAD) was calculated across the entire photometry trace during a given session. We conservatively estimated the area under the curve for each session, and then determined the percentage of that area that was above a threshold.

**Cell physiology.** To inhibit GPe-PV neurons that project to the midbrain, 500 nl of CAV2-FLEXloxP-Flp was injected into the SNr, and 300 nl of AAV-FLEXloxP-K2.2 was injected into the Gpe of Pvalb-Cre mice. All injections were bilateral. Animals were tested 2 weeks later.

**Histology.** To inhibit GPe-PV terminals in the midbrain, animals were injected with 300 nl of AAV-FLEXloxP-TeTLCxI or AAV-FLEXloxP-hMADi in the GPe, and a bilateral cannula guide (C235G, 26GA, C/C distance 1.8 mm, 5 mm pedestal, cut 3.5 mm below the pedestal, custom specified for mouse bilateral ventral midbrain coordinates, Plastics One) was implanted over the ventral midbrain (coordinates AP = 3.5, ML 0.9, DV = 3.13). Two skull screws were implanted for headstaple stability, and headcaps were sealed with Metabond and Geristore. CNO microspheres were synthesized to enable slow release of CNO after a single infusion. Degradase PLGA CNO microspheres were custom ordered from Phosphorex. Beads of target mean diameter 1 μm were dissolved in 0.5% trehalose at a concentration of 5 mg microsphere per ml. The estimated CNO loading efficiency was 5%, the estimated burst release was 50%, and the estimated release time was 7 days. The target concentration of CNO release at a steady state was 100 pg/h.

On day 1 of the behavioural protocols, animals were given an IP saline injection. On day 2, one hour before locomotor testing, all cannula guides were pierced with a dummy infuser (C235I, 33GA, project 1.5MM past guide), and mice were given an IP saline injection one hour later. On day 3, CNO microspheres were infused one hour before locomotor testing, 250 nl per side at 250 nl/min, and mice were given an IP saline injection one hour later and tested for effects on basal locomotion. On days 3–6, animals were run through the CPP protocol, and on days 6–10 (starting on the same afternoon) they were run through the LMS protocol. We then waited for CNO washout and repeated the same protocol. For experiments with subsequent infusions of CNO, we used a 2-day expression period for each infusion.

For manipulations of SNr-GABA neurons, 500 nl of AAV-FLEXloxP-hMADi was injected bilaterally into the SNr of GAD2-Cre mice, or 500 nl of AAV-FLEXloxP-hMADi was injected into the SNr and 300 nl of AAV-FLEXloxP-K2.2 was injected into the Gpe of GAD2-Cre-Pvalb-Flp animals. All injections were bilateral.

**Data analysis and statistics.** All statistics were calculated using GraphPad Prism 7 software. Statistical significance was assessed by paired t-tests with the exception of Fig. 2n, where a two-way ANOVA was used, and Figs 2k and 5m where a one-way ANOVA was used with the Geisser–Greenhouse correction followed by post hoc
Wilcoxon matched-pairs signed rank tests. Dot plots presented throughout the manuscript include a horizontal line representing the mean value for each group. In Fig. 1 and Extended Data Fig. 1, the length of each bar represents the mean. Error bars represent s.e.m. throughout. For all figures, NS \( P > 0.05 \), \( * P \leq 0.05 \), \( ** P \leq 0.01 \), \( *** P \leq 0.001 \), \( **** P \leq 0.0001 \).

**Data availability.** Numerical data for each figure are included with the manuscript as source data. All other data are available from the authors upon reasonable request.

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Extended Data Figure 1 | Changes to VTA-DA inputs induced by drugs of abuse. 

**a**, Quantification of monosynaptic inputs to VTA-DA neurons labelled in animals receiving single dose administration of cocaine, amphetamine, nicotine, morphine, saline, or fluoxetine one day before injection of RVdG into the VTA. Data were combined to generate Fig. 1c. 

**b**, Sample images of GPe neurons labelled by RVdG and co-stained for parvalbumin. Pie graph shows proportion of labelled cells that co-stained for parvalbumin.

**c**, Sample images of NAcMedS neurons labelled by RVdG in DAT-Cre;D1-tdTomato mice. Pie graph shows proportion of labelled cells that were D1+ as defined by presence of tdTomato (scale bars, 50 μm).

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Extended Data Figure 2 | Axonal projections of GPe-PV neurons.

a. AAV-FLEXloxP-mGFP was injected into the GPe of Pvalb-Cre animals, and mGFP⁺ axons were quantified throughout the brain. b. Quantification of fraction of mGFP⁺ axons in the indicated brain regions. c. Sample image of mGFP⁺ axons in the ventral midbrain (scale bar, 500 μm).

d. Quantification of fraction of mGFP⁺ axons in the indicated ventral midbrain brain regions. The schematics of the mouse brain in this figure were adapted from ref. 33. DLStr, dorsolateral striatum; DMStr, dorsomedial striatum.
Extended Data Figure 3 | Inhibition of GPe-PV neuron activity modestly affects cocaine-induced locomotion. a, Cre-dependent AAVs expressing YFP, hM4Di, K$_{v}$2.1 or TeTxLc were injected into the GPe of Pvalb-Cre animals. b, Quantification of effects of CNO on basal locomotion in animals expressing YFP or hM4Di (compared to YFP + saline: YFP + CNO, $P = 0.36$; hM4Di + CNO, $P = 0.59$).

c, Quantification of basal locomotion during GPe-PV neuron inhibition (hM4Di, $P = 0.37$; K$_{v}$2.1, $P = 0.66$; TeTxLc, $P = 0.27$). d, Quantification of cocaine-induced locomotion during GPe-PV neuron inhibition (hM4Di, $P = 0.37$; K$_{v}$2.1, $P = 0.12$; TeTxLc, $P = 0.002$). The schematics of the mouse brain in this figure were adapted from ref. 33.
DAT-Cre mice

Extended Data Figure 4 | Labelled GPe inputs to the VTA correlated with LMS. a, AAV-FLExloxP-TC and AAV-FLExloxP-G were injected into the VTA of DAT-Cre mice. Eleven days later, animals were habituated for two days to an open field chamber, and given a drug injection the following day. RVdG was injected one day after the drug. Five days after RVdG injection, the animal was given a second injection of the same drug in the open field. b, Normalized labelled GPe inputs plotted against the relative locomotion in session 2 vs. session 1 for cocaine ($n=34$), amphetamine ($n=5$), nicotine ($n=5$) and morphine ($n=5$). Regression line is plotted for all drugs combined. c–e, Labelled GPe inputs after a single dose of cocaine significantly correlated with LMS (c) but not total locomotion after the first (d) or second (e) dose of cocaine. f–h, Plots of labelled GPe inputs vs. LMS for amphetamine (f; 1 mg kg$^{-1}$), nicotine (g; 0.5 mg kg$^{-1}$), or morphine (h; 10 mg kg$^{-1}$). The schematics of the mouse brain in this figure were adapted from ref. 33.
Extended Data Figure 5 | Inhibition of the GPe prevents morphine LMS and CPP. 

**a**. A Cre-dependent AAV expressing either YFP or hM4Di was injected into the GPe of Pvalb-Cre mice. 

**b, c**. Quantification of LMS (b; \( P = 0.022 \)) and CPP (c; \( P = 0.0005 \)) in animals in which YFP or hM4Di (activated by CNO) were expressed in GPe-PV neurons. The schematics of the mouse brain in this figure were adapted from ref. 33.
Extended Data Figure 6 | Inhibition of GPe-PV→midbrain neurons that project to the midbrain blocks cocaine-induced CPP and LMS. a, CAV-FLExloxP-Flp was injected into the ventral midbrain, and AAV-FLExFRT-Kir2.1 or AAV-FLExFRT-YFP was injected into the GPe of Pvalb-Cre mice.

b, c. Quantification of LMS (b; $P = 0.019$) and CPP (c; $P = 0.0067$) in animals in which YFP or Kir2.1 were expressed in GPe-PV neurons projecting to the ventral midbrain. The schematics of the mouse brain in this figure were adapted from ref. 33.
Extended Data Figure 7 | GPe-PV neurons that project to the midbrain collateralize to multiple subcortical targets. a, CAV-FLExloxP-Flp was injected into the ventral midbrain, and AAV-FLExFRT-mGFP was injected into the GPe of Pvalb-Cre mice. b, Representative image of mGFP+ collaterals in the thalamus and subthalamic nucleus (STN) (scale bar, 500 μm). c, Quantification of projection fraction of collaterals to indicated target regions. The schematics of the mouse brain in this figure were adapted from ref. 33.
Extended Data Figure 8 | Dopamine neuron activity is required for the development of LMS and CPP. a, Breeding scheme for experiments. LSL, loxP stop loxP. b, c, Quantification of LMS (b; $P = 0.001$) and CPP (c; $P = 0.005$) in control animals or animals expressing hM4Di in dopamine neurons receiving CNO.
Extended Data Figure 9 | Map of anatomical location of ventral midbrain cells from which whole cell recordings were made. Individual dots indicate location of cells in which ChR2-evoked IPSCs due to ChR2 expression in GPe-PV neurons could be detected (connected) or not (not connected) in NAcLat-projecting (a) or NAcMed-projecting (b) VTA-DA cells, and SNr-GABA (c) or VTA-GABA (d) cells. The schematics of the mouse brain in this figure were adapted from ref. 33.
Extended Data Figure 10 | GPe-PV neurons mediate their effects through SNr-GABA neurons. a, Procedure to test LMS and CPP during SNr-GABA activation. b, c, Activating SNr-GABA neurons with CNO prevented LMS (b; $P = 0.010$) and CPP (c; $P = 0.015$). d, Injection strategy to test whether SNr-GABA neurons are downstream of GPe-PV neurons. e, f, While expression of Kir2.1 in GPe-PV neurons prevented LMS and CPP, this suppression was overcome by concurrent inhibition of SNr-GABA neurons (e; $P = 0.035$, f; $P = 0.036$). The schematics of the mouse brain in this figure were adapted from ref. 33.
# Life Sciences Reporting Summary

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## Experimental design

1. **Sample size**
   - Describe how sample size was determined.
   - No statistical methods were used to predetermine sample size.

2. **Data exclusions**
   - Describe any data exclusions.
   - Data were excluded only in experiments where viral injections were performed and post-hoc analysis of viral targeting demonstrated that injections were inaccurate.

3. **Replication**
   - Describe whether the experimental findings were reliably reproduced.
   - Results described throughout the paper were reproduced. Multiple rounds of experimentation were required, and multiple methods were used where applicable (e.g., multiple drugs of abuse show similar increases in rabies-labeled GPe inputs, Extended Data Fig. 1, and multiple methods of GPe inhibition all block LMS and CPP, Fig. 4). No issues were identified in replicating any of the reported findings.

4. **Randomization**
   - Describe how samples/organisms/participants were allocated into experimental groups.
   - Experiments described in Fig.’s 1, 2a-h and 2l-n, 3, 5g-j and Extended Data Figs. 1a, 4, 5, 6, 8, and 10 were randomized and investigators were blinded to allocation and outcome assessments; all other experiments were not randomized and investigators were not blinded.

5. **Blinding**
   - Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   - Experiments described in Fig.’s 1, 2a-h and 2l-n, 3, 5g-j and Extended Data Figs. 1a, 4, 5, 6, 8, and 10 were randomized and investigators were blinded to allocation and outcome assessments; all other experiments were not randomized and investigators were not blinded.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.
6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

| n/a | Confirmed |
|-----|-----------|
| ☑   | The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.) |
| ☑   | A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |
| ☑   | A statement indicating how many times each experiment was replicated |
| ☛   | The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section) |
| ☛   | A description of any assumptions or corrections, such as an adjustment for multiple comparisons |
| ☛   | The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted |
| ☛   | A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range) |
| ☛   | Clearly defined error bars |

See the web collection on statistics for biologists for further resources and guidance.

7. Software

Describe the software used to analyze the data in this study.

MATLAB software was used to analyze fiber photometry data. All electrophysiology data were collected using Axograph software. GraphPad Prism was used to analyze all other data.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

There are no restrictions on materials.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

The following primary antibodies were used: Rat anti-mCherry, Life Sciences, 1:2000; chicken anti-GFP, Aves Labs, 1:1000; rabbit anti-TH, Millipore, 1:1000; Goat anti-Fos, Santa Cruz, 1:500. The following secondary antibodies were used from Jackson ImmunoResearch at a concentration of 1:250: donkey anti-chicken AlexaFluor488; donkey anti-goat AlexaFluor488; donkey anti-rat AlexaFluor555; donkey anti-goat AlexaFluor555; donkey anti-rabbit AlexaFluor647.

Antibodies were validated 1. In the case of an extensively published antibody, by testing the specificity of staining to the appropriate regions (e.g. tyrosine hydroxylase, TH), or 2. by comparing between animals (e.g. cFos) and noting labeling that is above background but not consistent with non-specific staining.
10. Eukaryotic cell lines
   a. State the source of each eukaryotic cell line used. N/A
   b. Describe the method of cell line authentication used. N/A
   c. Report whether the cell lines were tested for mycoplasma contamination. N/A
   d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use. N/A

- Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals
   Provide details on animals and/or animal-derived materials used in the study.
   Mice were housed on a 12-hour light/dark cycle with food and water ad libitum. Males and females from a mixed CD1 and C57/BL6 background were used for all experiments in approximately equal proportions. All surgeries were done under isoflurane anesthesia. All procedures complied with the animal care standards set forth by the National Institute of Health and were approved by Stanford University’s Administrative Panel on Laboratory Animal Care and Administrative Panel of Biosafety.

Policy information about studies involving human research participants

12. Description of human research participants
   Describe the covariate-relevant population characteristics of the human research participants.
   N/A