Phencyclidine and methamphetamine cause cognitive deficits by changing pyramidal neuron transmitter identity in the prefrontal cortex

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ABSTRACT

Decreased cognitive ability is a major consequence of exposure to drugs of abuse, but the underlying neuroplastic changes have been elusive. We show that both phencyclidine and methamphetamine cause a population of prelimbic pyramidal neurons to switch from a glutamatergic to a GABAergic phenotype. Overriding the gain of GABA with RNA-interference prevents drug-induced cognitive deficits and locomotor sensitization, connecting the change in neurotransmitter identity with altered behavior. Chemogenetic suppression of drug-induced hyperactivity also prevents the change in transmitter phenotype or reverses it after it has occurred, preventing or rescuing the associated behavioral changes. These findings may provide therapeutic opportunities to mitigate drug-induced cognitive deficits by manipulating electrical activity in the prelimbic cortex.

MAIN

Repeated exposure to drugs of abuse alters behavior by causing synaptic plasticity in brain circuits that regulate reward and cognitive processing (*1-4*). Drug-induced alterations in prefrontal cortex activity and function have been linked to the appearance of cognitive deficits that persist during abstinence and can contribute to relapse (*3, 5, 6*). Exposure to drugs also increases the number of neurons expressing a specific transmitter

in subcortical regions, promoting nicotine and alcohol preference (*7*), withdrawal symptoms (*8*) and drug-seeking (*9*). Neurons can change their neurotransmitter identity when external stimuli increase neuronal activity, often switching from an excitatory to an inhibitory transmitter or *vice versa*, causing changes in behavior (*10, 11*). Evidence that drugs increase the number of neurons expressing a specific transmitter (*7-9*) suggests that changes in the transmitter phenotype of subpopulations of neurons could also be a form of plasticity underlying drug-induced changes in behavior. However, whether this form of plasticity is involved in drug-induced cognitive deficits remains an open question.

RESULTS

Drugs of abuse change the neurotransmitter identity of prelimbic cortex neurons

We investigated whether sustained exposure to phencyclidine (PCP) or to methamphetamine (METH) causes neurons to change their neurotransmitter identity. We analyzed the medial prefrontal cortex because of its relevance for drug-induced cognitive deficits (*3, 5, 6*) and focused on the prelimbic subregion (PrL). Because changes in transmitter identity are activity-dependent (*10, 12, 13*), we first determined whether drug administration alters c-fos expression in the PrL. Both PCP and METH increased PrL neuronal activity after a single injection as well as throughout a 10-day treatment (fig. S1, A to D), suggesting that changes in transmitter identity could occur in this region upon exposure to either drug. To learn if this was the case, we examined pyramidal neurons, which represent the largest population in the PrL (*14*) and can be identified by expression of the vesicular glutamate transporter 1 (VGLUT1). We used VGLUT1-Cre+/-::H2BmCherry+/- mice to permanently label pyramidal neurons with nuclear mCherry fluorescence, thus allowing their identification following changes in gene expression or transmitter profile (Fig. 1A). In the PrL of saline-treated controls, we detected ~600 mCherry+ neurons co-expressing GABA as well as its synthetic enzyme, glutamic acid decarboxylase 67 (GAD67). The number of mCherry+/GABA+, and mCherry+/GAD67+ neurons was doubled in PCP- and in METH-treated mice (Fig. 1, B and C, and fig. S2C) while the total number of PrL mCherry+ neurons did not change (fig. S2, A and B). These results suggested that drug exposure induces the synthesis and expression of GABA in a group of PrL pyramidal neurons not previously expressing this transmitter. PCP-

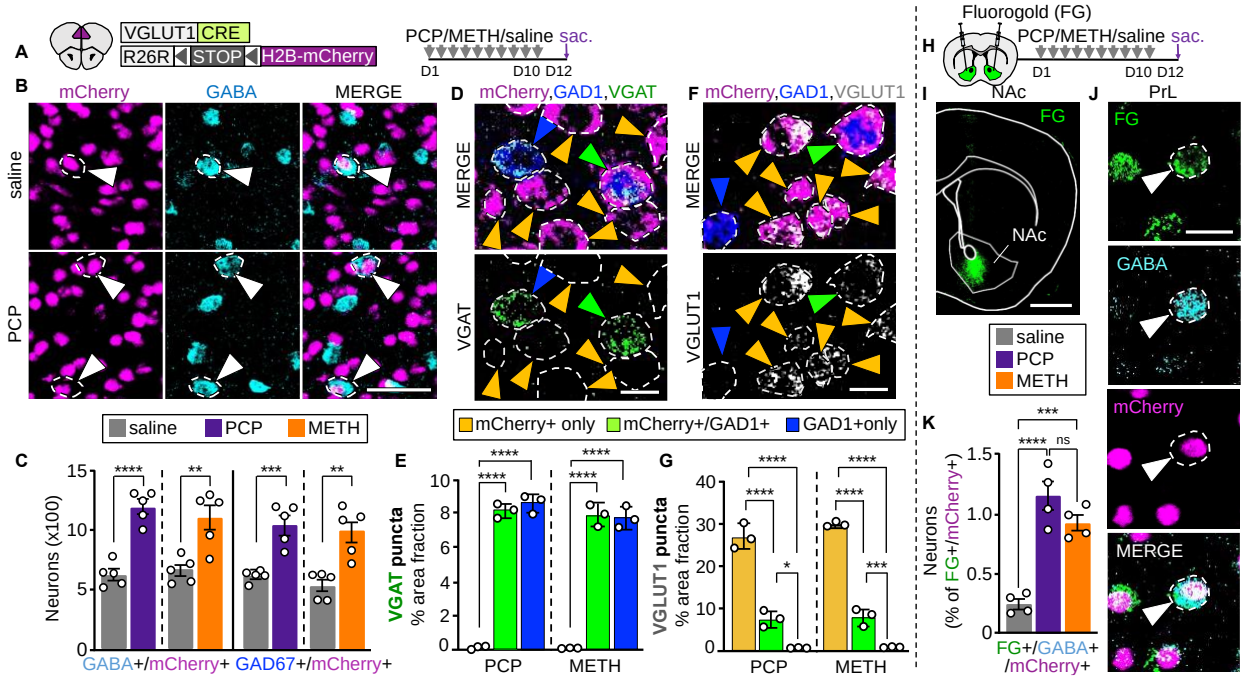

Fig. 1. Repeated exposure to either PCP or METH changes the transmitter identity of PrL pyramidal neurons. (**A**) Experimental protocol. (**B**) PrL neurons co-expressing mCherry and GABA (arrowheads). (**C**) Quantification of neurons co-expressing mCherry and GABA or GAD67. (**D**, **F**) Expression of VGAT (**D**) and VGLUT1 (**F**) mRNA in PrL neurons of PCP-treated mice. (**E**, **G**) Quantification of VGAT (**E**) and VGLUT1 (**G**) expression (measured as % of cell area occupied by mRNA puncta) across PrL neuronal subtypes. (**H**) Experimental protocol. (**I**) Fluoro-gold (FG) injection site in the NAc. (**J**) FG labeling of mCherry+/GABA+ PrL neuron. (**K**) Quantification of FG-expressing mCherry+/GABA+ neurons across treatments. Scale bars, (B) 50 μm, (D) and (F) 10 μm, (I) 1 mm, (J) 20 μm. Statistical significance (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001) was assessed using unpaired t-test or Mann Whitney test (**C**) and one-way ANOVA followed by Tukey's test (**E, G, K**). Data are mean ± SEM. Further statistical details are presented in Table S1.

exposure also causes a decrease in the number of PrL parvalbumin-positive (PV+) interneurons expressing the GABA synthetic enzyme GAD67 (fig. S3, A to C), in line with previous findings (*15*).

We then tested whether the expression of the GABA vesicular transporter (VGAT) and VGLUT1 was altered in pyramidal neurons that co-expressed or gained GABA. We used RNAscope to detect transcripts for mCherry, for the GABA synthetic enzyme (GAD1) and for either VGAT or VGLUT1 on PrL sections from PCP-, METH-, and saline-treated mice. We selectively decreased the amplification level for VGAT and VGLUT1 to obtain punctate staining that enabled detection of changes in expression levels (Fig. 1, D and F). Across treatments, pyramidal neurons that co-expressed or gained GABA

(GAD1+/mCherry+) also expressed the VGAT at the level of mCherry- GABAergic neurons (GAD1+ only) (Fig. 1, D and E, and fig. S4, A and B). At the same time, the expression level of VGLUT1 in mCherry+/GAD1+ neurons decreased by two-thirds compared to that of mCherry+ only pyramidal cells (Fig. 1, F and G, and fig. S4, A and C). These results revealed that both the pyramidal neurons that gained GABA after drug-exposure, as well as those expressing GABA in drug-naïve conditions, are characterized by high levels of GABAergic markers and low levels of VGLUT1. The subset of PrL mCherry+/GAD1+ neurons examined with RNAscope (100 neurons/animal) was twice as dense in PCP- and METH-treated mice compared to controls (fig. S4, D and E), mirroring the finding that drug exposure doubles the number of PrL mCherry-labelled neurons co-expressing GAD67 (Fig. 1C).

PCP and METH exert a strikingly similar effect on the neurotransmitter identity of PrL pyramidal neurons, leading us to ask whether the cells affected by each drug project to the same downstream target. We investigated the nucleus accumbens (NAc), since this region receives substantial glutamatergic innervation from the PrL and both the NAc and the PrL are involved in modulating behaviors that are affected by repeated intake of PCP or METH (*16-26*). We injected fluoro-gold (FG) into the NAc of VGLUT1-Cre+/-::H2BmCherry+/- mice and screened the PrL for mCherry+/GABA+ neurons expressing the retrograde tracer after exposure to PCP, METH or saline (Fig. 1H). In both PCP- and METH-treated mice, we detected mCherry+/GABA+ neurons labelled by FG, which accounted for ~1% of the total number of FG-labelled neurons. Such cells were rare in saline-treated controls (~0.2% of total number of FG-labelled neurons) (Fig. 1, H to K). While treatment with PCP or METH doubles the number of mCherry+/GABA+ neurons in the PrL of drug-treated mice compared to controls, the number of mCherry+/GABA+ PrL neurons that project to the NAc was 4-5 times higher in PCP- or METH- vs saline-treated mice (fig. S2D). This result indicates that most of the PrL mCherry+/GABA+ neurons projecting to the NAc have gained GABA upon drug-exposure, rather than coexpressing it in drug-naïve conditions. Thus, the NAc receives innervation from PrL neurons that have changed their transmitter identity after exposure to either drug, raising the possibility that both PCP and METH may affect the same cells.

The change in transmitter identity is mediated by drug-induced PrL hyperactivity

Demonstration that both PCP and METH had the same effect on the transmitter phenotype of PrL pyramidal neurons, despite activation of different receptors, prompted a question about their shared mechanism of action. Could the neurochemical change observed in PrL pyramidal neurons after exposure to PCP or METH be mediated by similar drug-induced changes in PrL activity? If this hypothesis were correct, suppressing PrL hyperactivity during drug treatment would prevent the change in transmitter identity. We first investigated whether PCP and METH differentially affect the activity of distinct neuronal cell types in the PrL. We found that both drugs increased c-fos expression specifically in PrL pyramidal neurons but not in non-glutamatergic cells (fig. S1, E and G). Because PrL pyramidal cells receive perisomatic inhibition from PV+ inhibitory interneurons in this region, which do not show changes in c-fos expression after administration of PCP or METH (fig. S1, F and H), we reasoned that hyperactivity of pyramidal neurons could be suppressed by chemogenetic activation of PV+ neurons. We expressed the chemogenetic receptor PSAML^{141F,Y115F}-5HT3HC (using AAV-syn::FLEX::PSAML141F,Y115F:5HT3HC-IRES-GFP) in the PrL of PV-Cre+/- mice, which enabled chemogenetic activation of transfected neurons upon administration of the PSEM^{308} ligand (fig. S5A) (*27*). We validated this approach by injecting PSEM^{308} immediately before an acute injection of either PCP, METH or saline (fig. S5B). After an injection of PSEM^{308}, c-fos expression was increased in neurons infected with the virus and identified by GFP, consistent with their expected chemogenetic activation (fig. S5, C and D). This strategy suppressed PCP- and METH-induced increase of c-fos expression specifically in PrL GABA- neurons (Fig. 2, A to C, and fig. S5E), thus demonstrating that it could be used to test the link between pyramidal neuron hyperactivity and change in transmitter identity.

We now tested the effect of combining chemogenetic activation of PV+ interneurons with drug-administration for the entire duration of drug treatment. PSEM^{308} was injected immediately prior to and 3h after each of 10 daily injections of either PCP, METH or saline (Fig. 2D). At the end of treatment, we quantified the number of glutamatergic neurons that had gained GABA using RNAscope to detect colocalization of

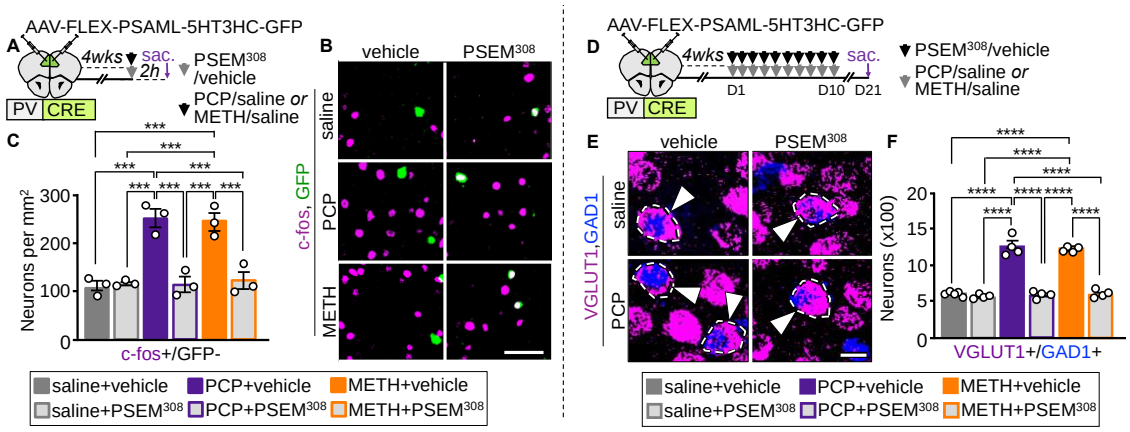

Fig. 2. Suppression of drug-induced PrL hyperactivity blocks the change in transmitter phenotype. (**A**) Experimental protocol. (**B**) c-fos+ (magenta) and GFP+ (green) cells following simultaneous injection of saline or drug and PSEM^{308} or vehicle (compare Fig.S1B). (**C**) Quantification of neurons expressing c-fos but not GFP in the PrL. (**D**) Experimental protocol. (**E**) PrL neurons co-expressing VGLUT1 and GAD1 (arrowheads) following saline/drug treatment combined with chemogenetic activation of PV+ neurons. (**F**) Quantification of neurons co-expressing VGLUT1 and GAD1. Scale bars, (B) 50 μm, (E) 20 μm. Statistical significance (***P<0.001, ****P<0.0001) was assessed using two-way ANOVA with Tukey's multiple-comparisons test. All data are presented as mean ± SEM. Further statistical details are presented in Table S7.

GAD1 and VGLUT1 mRNA. Since VGLUT1 expression is decreased but not completely lost in neurons that have changed their transmitter identity, we identified these cells by increasing the level of amplification of VGLUT1 RNA signal. The number of VGLUT1+/GAD1+ neurons was decreased in PCP- and METH-treated mice that received PSEM^{308} versus mice that did not, and was comparable to that of saline-treated controls (Fig. 2, E and F). These results indicated that suppressing drug-induced PrL hyperactivity is sufficient to prevent glutamatergic neurons from switching their neurotransmitter identity and suggested that drug-induced PrL hyperactivity could be the cause of the change in pyramidal neuron transmitter phenotype.

The change in transmitter phenotype affects behavior

We next investigated whether PrL glutamatergic neurons that gain a GABAergic phenotype are involved in the appearance of drug-induced cognitive deficits. We focused on impairments in episodic and working memory, since these behaviors are affected by repeated exposure to PCP or METH (*18-20, 25*) and are regulated by the PrL and NAc (*16, 17, 19, 23*). We also evaluated locomotor sensitization after PCP- and METH-exposure, as a measure of altered prefrontal function (*21, 22, 26*). To test if drug-induced

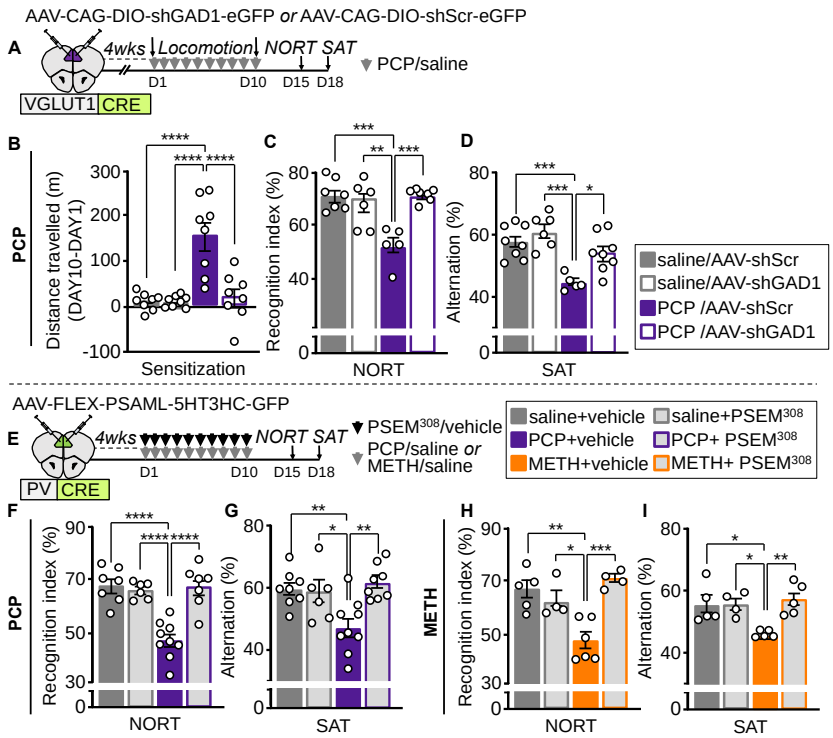

Fig. 3. Expression of shGAD1 in pyramidal neurons and chemogenetic activation of PV+ neurons each prevent drug-induced changes in behavior. (**A**) Experimental protocol. (**B-D**) shGAD1 prevents PCP-induced deficits in locomotor sensitization and in the NORT and SAT. (**E**) Experimental protocol. (**F-I**) Chemogenetic activation of PV+ neurons to suppress pyramidal neuron activity prevents both PCP- and METH-induced deficits in the NORT and SAT. Statistical significance (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001) was assessed using two-way ANOVA with Tukey's multiple-comparisons test. All data are presented as mean ± SEM. Further statistical details are presented in Table S9.

gain of GABA is sufficient to influence behavior, we first investigated the behavioral effects of PCP-treatment on mice when GAD1 expression in PrL glutamatergic neurons had been selectively suppressed. VGLUT1-Cre+/- mice were injected in the PrL with a Cre-dependent AAV expressing shRNA for GAD1 (AAV-DIO-CAG-shGAD1-GFP)(*10*), or an AAV-CAG-DIO-shScr-GFP (expressing scrambled shRNA) as control, before being exposed to PCP- or saline treatment (Fig. 3A and fig. S6, A and B). shGAD1 suppressed GABA expression in transfected neurons of both PCP- and saline-treated mice (fig. S6, C and D), indicating that GAD67 is the main enzyme for GABA synthesis in pyramidal cells. While shGAD1 did not affect PCP-induced hyperlocomotion on the first day of treatment (fig. S7, A and B), it prevented the appearance of locomotor sensitization after a 10-day PCP-exposure (Fig. 3B and fig. S7, A to C), indicating that PCP-induced gain of GABA is required for sensitization to the acute locomotor effect of the drug. Furthermore, shGAD1 prevented the appearance of PCP-induced impairment of object recognition memory in the novel object recognition test (NORT), without affecting the behavioral performance of

saline-treated mice (Fig. 3C). Similarly, shGAD1-injected mice did not show PCP-induced spatial working memory deficits in the spontaneous alternation task (SAT) (Fig. 3D). In both tests, drug- and/or shGAD1 did not change exploratory behaviors, as shown by the absence of difference across treatments in the time required to reach the criterion of 20s object exploration in the NORT (fig. S7D), and in the number of arm entries in the SAT (fig. S7E). The observation that shGAD1 did not affect the behavioral performances of saline-treated control mice (Fig. 3, B to D, and fig. S7, B to E) suggests that glutamatergic neurons gaining GABA upon PCP-exposure, but not those co- GABA before drug-exposure, are important in mediating drug-induced locomotor sensitization and memory impairments in the NORT and the SAT. Overall, these data indicate a causal link of PCP-induced gain of GABA to behavioral alterations.

We now tested whether blocking the change in transmitter phenotype through chemogenetic activation of PV+ inhibitory neurons prevents the alteration of these behaviors (Fig. 3, E to I, fig. S8, B and C, and fig. S9, B and C). Remarkably, suppression of drug-induced PrL hyperactivity prevented both PCP- and METH-induced appearance of memory deficits in the NORT, as well as in the SAT (Figure 3, F to I), without influencing exploratory behaviors (fig. S8, A, D, and E, and fig. S9, A, D, and E). We also found, in mice treated with PSEM^{308}, that drug-induced hyperlocomotion was suppressed on both the first and last day of treatment (fig. S8, A to C, and fig. S9, A to C). This effect is not linked to the gain of GABA, which has not yet occurred on the first day of treatment, but can be explained by the ability of chemogenetic stimulation to suppress acute drug-induced hyperactivity of PrL glutamatergic neurons (*28*). Across experiments, PSEM^{308} did not affect the behavior of saline-treated controls, indicating that suppression of PrL activity specifically affects the behavioral performance of drug-treated mice.

Normalizing PrL neuronal activity after drug-exposure reverses the change in neurotransmitter identity and the associated behavioral alterations

We next determined whether the change in neurotransmitter identity can be reversed after the end of drug-exposure and thereby rescue the behavioral alterations. Other neurons that change their transmitter phenotype in response to a different stimulus spontaneously reverse to expression of their original transmitter within a week of

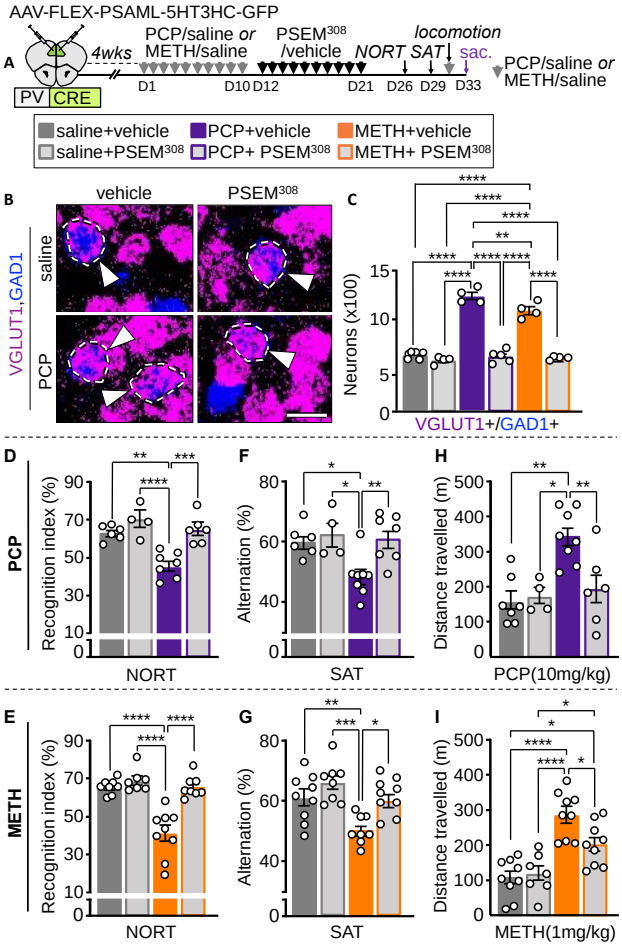

Fig. 4. Chemogenetic activation of PrL PV+ neurons reverses the drug-induced switch in pyramidal neuron transmitter identity and rescues the associated changes in behavior. (**A**) Experimental protocol. (**B**) Neurons co-expressing VGLUT1 and GAD1 (arrowheads) across treatment groups. (**C**) Drug-induced increase in the number of VGLUT1+GAD1+ neurons is reversed by chemogenetic activation of PV+ neurons. (**D, F, H**) PCP-induced deficits in the NORT and SAT and locomotor sensitization are reversed by sustained activation of PV+ neurons. (**E, G, I**) METH-induced deficits in the NORT, SAT, and locomotor sensitization are reversed by sustained activation of PV+ neurons. Scale bars, (B) 20 μm. Statistical significance (*P<0.05, **P < 0.01, ***P<0.001, ****P<0.0001) was assessed using two-way ANOVA with Tukey's multiple-comparisons test. All data are presented as mean ± SEM. Further statistical details are presented in Table S17.

cessation of the stimulus that originally induced the change (*10*). However, this is not the case for the PrL pyramidal neurons that change their transmitter upon exposure to PCP or METH, as they still express the GABAergic phenotype after three weeks of drug-washout (Fig. 4C).

Since PCP-induced deficits in the NORT are reversed by treatment with the antipsychotic drug clozapine (*18*), we tested whether repeated exposure to clozapine also reverses the PCP-induced change in PrL pyramidal neuron transmitter phenotype. In VGLUT1-Cre+/-::H2BmCherry+/- mice that received clozapine-treatment after PCP-exposure, the number of PrL mCherry+/GABA+ pyramidal neurons was reduced compared to that of mice treated with PCP alone and not different from that of saline-treated controls (fig. S10, A to C).

Clozapine did not affect the number of mCherry+/GABA+ neurons in saline-treated mice, indicating that this antipsychotic drug is effective in selectively reversing the PCP-

induced change in transmitter identity of the pyramidal neurons. As expected, clozapine treatment also rescued PCP-induced memory deficits in the NORT and SAT, without affecting the behavioral performance of mice that had not received PCP (fig. S10, D to J).

Clozapine interference with the acute PCP-induced increase in PrL c-fos expression (*29, 30*) made us question whether reversal of the gain of GABA is due to an effect on neuronal activity. To test this hypothesis, we first evaluated baseline PrL activity after the end of drug-treatment and detected a higher number of c-fos+ cells in PCP- and METH-treated mice compared to controls (fig. S11, A to C). Clozapine administration returned c-fos expression to baseline when administered after PCP treatment (fig. S12, A to D), suggesting that the increased level of neuronal activity after PCP-treatment is necessary to maintain the newly acquired transmitter phenotype. Because glutamatergic rather than non-glutamatergic PrL neurons show increased baseline expression of c-fos after the end of drug-exposure (fig. S11D), we tested whether neuronal activity can be returned to baseline by chemogenetic activation of PrL PV+ neurons. This chemogenetic intervention was sufficient to normalize c-fos expression in the PrL of PCP- and METH-treated mice (fig. S13, A to D), thus encouraging the use of this approach to investigate the role of neuronal activity in the maintenance of PrL pyramidal neuron transmitter phenotype.

We then asked whether repeatedly activating PrL PV+ neurons to suppress activity in pyramidal neurons, after the neurotransmitter phenotype has changed, is sufficient to reverse this change and the associated behavioral alterations. The transmitter phenotype of PrL pyramidal neurons in mice treated with either PCP or METH was indistinguishable from that in control animals after 10-day chemogenetic activation of PrL PV+ interneurons (Figure 4, A to C). This result demonstrates that PrL neuronal activity is involved in both the induction and in the maintenance of the transmitter switch. Activation of PrL PV+ interneurons rescued memory deficits in the NORT and SAT (Fig. 4, D to G, and fig. S14, A to C, and S15, A to C) and suppressed locomotor sensitization to both PCP and METH (Fig. 4, H and I, and fig. S14, A and D, and S15, A and D). These data show that suppressing drug-induced increase in PrL baseline activity following drug-exposure reverses the change in PrL neuron transmitter phenotype and the associated behavioral alterations.

DISCUSSION

Our findings reveal a novel mechanism by which sustained exposure to either PCP or METH causes cognitive deficits. Each drug induces a subset of PrL pyramidal neurons to acquire a GABAergic phenotype and lose VGLUT1. Overriding drug-induced gain of GABA in pyramidal neurons prevents the appearance of both locomotor sensitization and memory deficits in the NORT and SAT, thus establishing a causal link between the change in transmitter identity and these behavioral alterations. These findings are consistent with the modulation of these behaviors through the involvement of both the PrL and the NAc, which receives input from PrL neurons that have changed their transmitter identity (*16, 17, 19, 23, 24, 26*). The results align with other reports of addictive substances inducing changes in neurotransmitter expression in different brain regions (*7-9, 31, 32*). Evidence that these drug-induced changes in neurotransmitter identity affect behavior (*7-9*) indicates the importance of investigating them as a form of plasticity that can contribute to the escalation from recreational drug-use to addiction. The use of PCP and METH to study psychosis and schizophrenia like-symptoms in rodents (*33, 34*) suggests that our findings may also be relevant for these fields of research.

Our observation that PCP changes the neurotransmitter phenotype of PrL pyramidal neurons provides a new framework in which to interpret discrepancies between earlier studies. Some reports find no change in GAD67 expression in the whole PrL or frontal cortex of PCP-treated rodents (*35, 36*), while others describe a decrease in prefrontal cortex GAD1 mRNA levels (*37*) and loss of GAD67 by PrL PV+ interneurons (*15*). The gain of GAD67 by pyramidal neurons and its loss by PV+ interneurons (*15*) may balance each other, thus preventing detection of changes in GAD67 levels when the PrL is analyzed as a whole (*35, 36*). In line with this hypothesis, we found that PCP causes PrL pyramidal neurons to gain GABA, and decreases the number of GAD67+ PV+ interneurons in the PrL (fig. S3, A to C; (*15*)).

We observed the same change in PrL neurotransmitter identity in response to treatment with either PCP or METH. Since these drugs bind to distinct molecular targets and differ in their acute effects on the brain (*38, 39*), the change in the transmitter phenotype occurs downstream. Drug-induced PrL pyramidal neuron hyperactivity is a common denominator of the action of both PCP and METH, consistent with previous

reports of activity-dependent changes in transmitter identity (*10, 12, 13, 40, 41*). Suppressing drug-induced hyperactivity via chemogenetic activation of PV+ interneurons prevented the change in transmitter phenotype as well as the appearance of behavioral alterations, implying that PrL-hyperactivity is necessary to produce these changes. These results raise the possibility that similar changes are caused by other drugs that increase PrL activity, such as opioids (*42*) and tetrahydrocannabinol (*43*). Many addictive substances, including PCP (*44*) and METH (*38*), acutely promote dopamine (DA) release from ventral tegmental area (VTA) neurons, suggesting that DA could play a role in the drug-induced change in transmitter identity.

PrL pyramidal neurons maintained their newly acquired transmitter phenotype after the end of drug-treatment and PrL neuronal activity in drug-treated mice remained higher than in controls. Suppressing this elevated electrical activity with repeated chemogenetic activation of PrL PV+ neurons was sufficient to reverse PCP- and METH-induced changes in PrL pyramidal neuron transmitter phenotype and rescue the associated behavioral alterations. Exposing PCP-treated mice to chronic treatment with clozapine also reduced c-fos expression in the PrL of PCP-treated mice, as previously reported for acute clozapine administration (*29, 30*), and reversed the PCP-induced changes in transmitter phenotype and behavior. This effect may be mediated by increased inhibitory input to PrL pyramidal neurons, as suggested by a larger number of PFC c-fos+ GABAergic cells after acute clozapine treatment (*45*) and rescue of PCP-induced decrease in GAD67 and PV expression in PV+ neurons after chronic exposure (*15)*. It will be of interest to learn whether the induction or reversal of changes in transmitter identity contributes to the therapeutic effect of transcranial magnetic stimulation (*46-50*) and other forms of non-invasive manipulation of neuronal activity (*51*).

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