UNIVERSITY OF CALIFORNIA SAN DIEGO

Clozapine Reverses the Glutamate-to-GABA Neurotransmitter Switch Induced by Phencyclidine in the Prelimbic Cortex of Mice

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by

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The Thesis of Arth Thaker is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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ABSTRACT OF THE THESIS

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Arth Thaker

Master of Science in Biology

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Professor Nicholas Spitzer, Chair
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Neurotransmitter switching (NTS) is a form of neuroplasticity in which a neuron loses the neurotransmitter it was expressing and gains a new one in response to external stimuli causing sustained changes in brain activity. Preliminary data from our laboratory have demonstrated that phencyclidine (PCP), a drug of abuse also known as angel dust, induces a glutamate-to-GABA transmitter switch in prelimbic cortex pyramidal neurons, and that this switch in transmitter is causally linked to the appearance of PCP-induced cognitive deficits. Clozapine is an antipsychotic medication that prevents PCP-induced increase in prefrontal cortex activity and has been shown to rescue PCP-induced

cognitive deficits. In this thesis, I investigated whether clozapine administration can be used to reverse the NTS caused by PCP. To permanently label glutamatergic neurons, even when they change their transmitter identity, I used a mouse line expressing both VGLUT1-cre and a Cre-dependent mCherry reporter. Mice received daily injections of PCP or saline as a control for 10 days followed by an additional 2 weeks of treatment with either clozapine or vehicle as a control. After sacrifice, prelimbic cortex sections were immunostained for the inhibitory transmitter GABA and the number of neurons co-expressing mCherry and GABA across the experimental groups was counted. In mice treated with both PCP and clozapine the number of mCherry+/GABA+ neurons was significantly reduced as compared to that of mice treated with only PCP and was comparable to that of control. This result demonstrates that clozapine can successfully reverse the glutamate-to-GABA NTS induced by PCP.

INTRODUCTION

Neurotransmitter switching

Neurotransmitter switching (NTS) is a form of neuroplasticity in which a neuron gains the expression of a novel neurotransmitter and loses the expression of the one it was expressing before. This change in neurotransmitter phenotype occurs when an external stimulus alters electrical activity in the nervous system for a sustained period (Spitzer, 2015; Spitzer, 2017).

Early evidence of NTS was observed in cultures of dissociated superior cervical ganglion (SCG) neurons of neonatal rats (Patterson and Chun, 1974). Patterson and colleagues observed that, depending on the culture conditions, SCG neurons can assume either a noradrenergic or a cholinergic phenotype. For example, when SCG neurons were co-cultured with non-neuronal cells, they differentiated into cholinergic neurons. Instead, in the absence of non-neuronal cells, the SGC neurons assumed a noradrenergic phenotype (Patterson and Chun, 1974). This established that transmitter identity can be influenced by environmental factors.

Evidence for neurotransmitter switching *in vivo* appeared in a subsequent study of the development of neuronal innervation of the foot pad sweat glands in rats. Nerve terminals were initially rich in dense core synaptic vesicles containing catecholamines. However, these vesicles disappeared by 3 weeks of age when the neurons began exhibiting cholinergic features (Landis and Keefe, 1983). The observed change was not due to different populations of neurons existing simultaneously or competitive synaptogenesis since the pruning necessary for this extensive network to be replaced

was not detected at innervation sites. These results suggested that a single population of neurons is responsible for innervation of the sweat glands and switches neurotransmitter phenotype during development.

Later studies provided insights into the mechanisms behind NTS and demonstrated that neuronal activity can drive changes in neurotransmitter identity. Borodinsky and colleagues (2004) employed manipulation of ion channels and pharmacological agents to either enhance or suppress the activity of spinal neurons in developing *Xenopus* embryos. Using this strategy, they demonstrated that suppressing neuronal activity increases the expression of excitatory transmitters (i.e. glutamate and acetylcholine) and decreases the expression of inhibitory ones (i.e. GABA and glycine) in the developing neural tube. Changes in the opposite direction occurred instead when neuronal activity was increased. Overall, these findings suggest that alterations in regional neuronal activity guide the respecification of neurotransmitter phenotype during development.

More recent studies have investigated NTS in rodents. For example, when rats were exposed to a long-day photoperiod, which is stressful for them as nocturnal animals, the number of dopaminergic neurons in the paraventricular nuclei of the hypothalamus decreased while the number of neurons expressing somatostatin increased. In contrast, the opposite occurred in rats exposed to a short-day photoperiod (Dulcis *et al.,* 2013). Remarkably, while rats exposed to a long-day photoperiod showed anxiety and depressive-like behavior, rats exposed to a short-day photoperiod displayed low levels of anxiety and depression. These findings serve as one of the benchmarks for how

environmental stimuli can induce NTS in the adult rodent brain which is correlated with changes in behavior.

Extensive motor activity on a running wheel induces an acetylcholine-to-GABA neurotransmitter switch in the mouse pedunculopontine nucleus (PPN) (Li and Spitzer, 2020). C-fos is an immediate early gene whose expression is indicative of recent neuronal activity (Minciacchi *et al.,* 1993). Increased c-fos expression is detected in the PPN after running wheel activity and correlates with the appearance of NTS. After running, mice showed improvements in motor skill learning, measured by performance on the rotarod and balance beam. Significantly, overriding the transmitter switch with molecular tools prevented running-induced improvements in motor behavior, demonstrating that exercise leads to NTS from acetylcholine to GABA that causes enhancement of motor skill learning (Li and Spitzer, 2020). Overall, the results obtained by Dulcis and colleagues (2013) and by Li and Spitzer (2020) revealed that changes in transmitter identity can cause significant changes in behavior, which can be either detrimental or beneficial.

Drugs of abuse

Repeated exposure to drugs of abuse causes neuroplastic changes across the brain that influence behavior and can contribute to the development of addiction, which is defined as a chronic and relapsing illness characterized by the compulsion to seek and take the drug (Koob and Volkow, 2010). The transition from occasional drug use to addiction entails massive neuroplasticity in the brain circuits that regulate reward, impulse control, and mood (Koob and Volkow, 2010; Volkow and Morales, 2015). Remarkably, one of the effects of drug intake is to influence brain activity both acutely and chronically

(Goldstein and Volkow, 2011; Kargieman *et al.,* 2007; Castane *et al.,* 2015), suggesting that neurotransmitter switching may occur in response to drug intake and potentially contribute to the associated changes in behavior. However, studies investigating the potential involvement of NTS in the field of drug abuse and addiction are just starting to emerge. A first demonstration of NTS involvement in drug seeking and addiction recently appeared from the work of Romoli, Dulcis and colleagues (Romoli *et al.,* 2019). They showed that mice that were exposed to nicotine during the neonatal period, as adults, displayed both enhanced drug preference and, if re-exposed to nicotine, an increased number of TH+ cells in the VTA. This evidence suggested that neonatal exposure to nicotine primes the brain to be highly susceptible to drug consumption during adulthood (Romoli *et al.,* 2019).

The prefrontal cortex (PFC) is a region of the brain that displays changes in neuronal activity after drug intake (Goldstein and Volkow, 2011). Both pre-clinical and clinical studies show a clear increase in the activity of the prefrontal cortex after acute intake of psychostimulants (such as methamphetamine and cocaine) and other drugs like phencyclidine (PCP) (Goldstein and Volkow, 2011; Kargieman *et al.,* 2007). This raised the possibility that NTS can be induced in this region after exposure to these drugs.

Phencyclidine induces NTS that causes behavioral alterations

Based on the above evidence, our lab has been investigating whether PCP can cause NTS. PCP, also known as angel dust, is a dissociative anesthetic that acts as a NMDA receptor antagonist (Clarke, 2007) and causes hyperactivity of pyramidal neurons

in the PFC by inhibiting activation of PV interneurons when acutely administered (Suzuki *et al.,* 2002; Kargieman *et al.,* 2007). Studies from different laboratories have shown that repeated administration of PCP causes cognitive deficits that can be detected in the novel object recognition test (NORT) and the spontaneous alternation task (SAT) (Castane *et al.,* 2015; Mouri *et al.,* 2007). The NORT measures memory retention and consists of two separate sessions. During the first session, the mouse is exposed to two identical objects and allowed to explore them for 10 min. The second session takes place after one day. At this point the mouse is presented with one of the objects from the previous day (generally labeled the “familiar object”) and a new different object (generally labeled the “novel object”). Mice have an exploratory instinct that drives them to spend time exploring the novel object, while disregarding the familiar one. However, this does not occur when memory is impaired. In this situation the mouse is unable to properly discriminate between the two and spends an equal amount of time interacting with the novel and the familiar objects. The SAT is used as a measure of working memory performance. During this test, the mouse is placed in a Y or T maze and allowed to freely explore. The number of times the mouse alternates between different arms of the maze (i.e. chooses to enter in a new arm without going back to the arm most recently visited) is used as a measure of working memory performance.

Data from our lab have shown that daily exposure to PCP for a period of 10 days induces NTS from glutamate to GABA in glutamatergic neurons in the prelimbic cortex (PrL) (Pratelli *et al.,* 2019; Pratelli *et al.,* 2020). To determine whether PrL glutamatergic neurons change their neurotransmitter phenotype after PCP exposure, glutamatergic neurons were permanently labeled using a genetic mouse model that expresses Cre

recombinase under the control of the promoter for the glutamate transporter VGLUT1 and Cre-dependent nuclearly-localized mCherry reporter (**Fig. 1A**). After exposure to either PCP or saline as a control, mice were sacrificed, and the brain was processed with immunohistochemistry to label the inhibitory neurotransmitter GABA. Using this approach, the expression of mCherry and GABA within the same cells provided evidence that a glutamatergic or previously glutamatergic neuron has gained GABA.

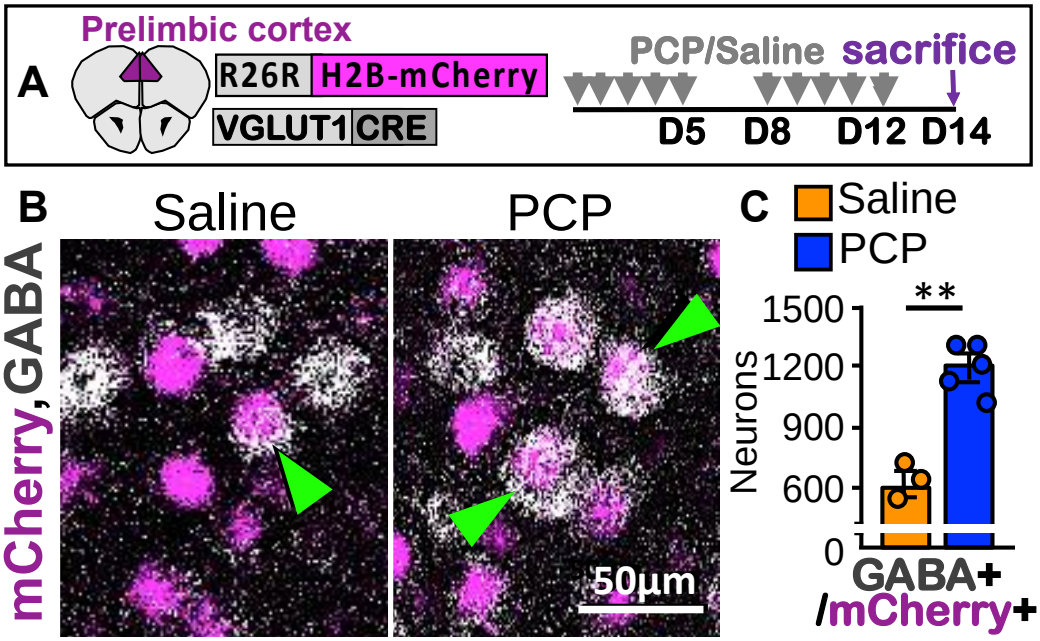

Figure 1: Repeated exposure to PCP induced a glutamate-to-GABA NTS in the PrL. **A.** Mice genotype and experimental timeline for PCP treatments. **B.** Representative images show that the number of glutamatergic mCherry+/GABA+ (green arrowheads) is higher in PCP-treated mice compared to saline-treated controls. **C**. Cell-counting shows that mCherry+/GABA neurons are nearly doubled in PCP-treated mice compared to controls. **, p<0.01, ***, p<0.001, n.s.= not significant.

In this way, our lab showed that although some neurons co-express mCherry and GABA in the saline-treated controls, their number doubles in mice that were treated with PCP for 10 days, demonstrating that a NTS has occurred (Pratelli *et al.,* 2021, **Fig. 1**).

Interestingly, when PCP-induced NTS was prevented (**Fig. 2**) the associated cognitive deficits in the NORT and SAT were rescued (Pratelli *et al.,* 2021; Pratelli *et al.,* 2020). To prevent PCP-induced NTS, VGLUT1-Cre +/- mice were injected in the PrL with an adeno-associated viral vector (AAV) expressing a GAD1shRNA RNA interference construct in Cre-dependent manner.

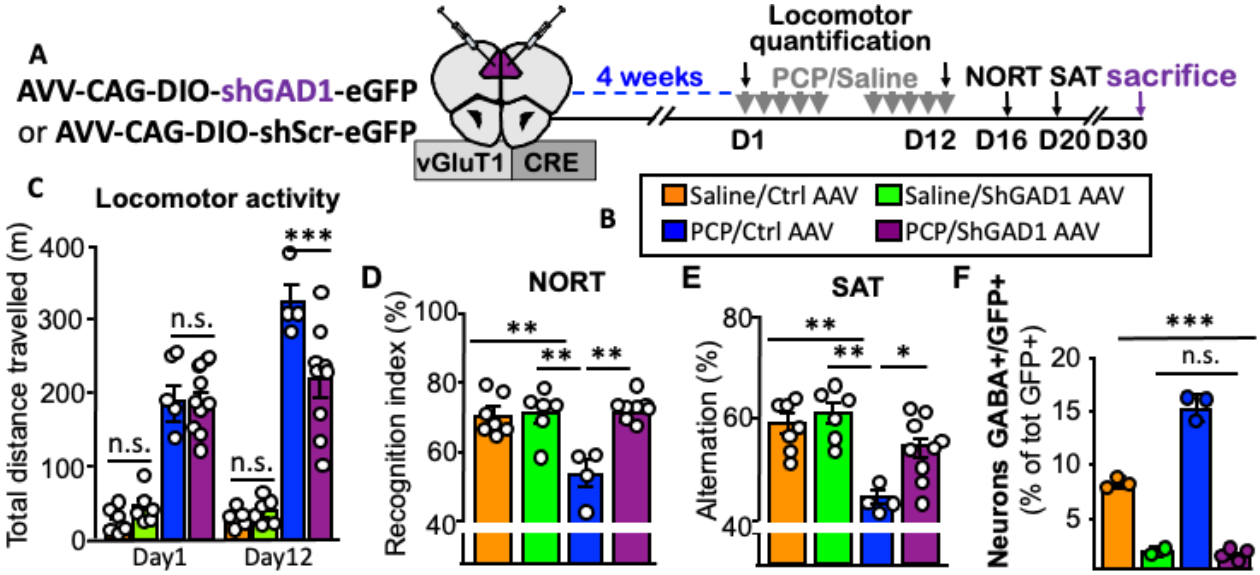

Figure 2: PCP-induced glutamate-to-GABA switch is causally linked to behavioral alterations. A. Experimental timeline. **B.** Treatment legend. **C.** PCP-induced locomotion measured on the first day (Day1) or the last day (Day12) of PCP administration. **D.** Graph shows mice performance in the NORT that quantifies memory retention using a recognition index (Recognition index % = *[time exploring novel object/(time exploring familiar object + time exploring novel object)x100*). **E.** Mice performance in the SAT that measures working memory as percentage of alternation (Alternation % = *[(alternations between arms/total arm entries) x100]*). **F.** Graph quantifying the number of glutamatergic neurons (identified by GFP expression) co-expressing GABA across the experimental groups. *, p<0.05 **, p<0.01, ***, p<0.001

Since GAD1 is the gene that codes for GAD67, the enzyme responsible for GABA synthesis, interfering with GAD1 expression prevents synthesis of GABA. The evidence that overriding the gain of GABA in switching neurons is sufficient to prevent the appearance of PCP-induced cognitive deficits was crucial, as it revealed the existence of a cause-and-effect relationship between the switch and the behavioral alterations.

Clozapine's effect on PCP-induced cognitive deficits

Clozapine is an atypical antipsychotic used to treat psychiatric disorders such as schizophrenia and psychosis (Khokhar *et al.,* 2018). Previous studies have shown that treatment with clozapine rescues the behavioral deficits in the NORT caused by previous exposure to PCP (Hashimoto *et al.,* 2005) as well as PCP-induced reversal-learning impairments (Idris *et al.,* 2005). At the same time, clozapine has been shown to reverse PCP-induced hyperexcitability of pyramidal neurons in the PFC (Kargieman *et al.,* 2007). Because our data establish a causal relationship between NTS and PCP-induced cognitive deficits (Pratelli *et al.,* 2019; Pratelli *et al.,* 2020), the ability of clozapine to reverse the same behavioral deficits and its effect on PCP-induced prefrontal cortex hyperactivity led us to hypothesize that clozapine may influence NTS as well.

In my research I have tested if clozapine can reverse the glutamate-to-GABA NTS induced by PCP. For this purpose, mice were treated with PCP daily for 10 days and, after 2 days of abstinence, were injected once a day with clozapine for 2 additional weeks. We have used 3 experimental groups: saline + vehicle, PCP + vehicle and PCP + clozapine. An additional control (saline + clozapine, needed to assess if clozapine itself can interfere with neurotransmitter expression in the PrL) is still being analyzed and has therefore not been included in this thesis. To investigate the ability of clozapine to reverse the NTS, I counted the number of switched neurons in the PrL across the different experimental groups. The results show that, consistent with our hypothesis, clozapine treatment is sufficient to reverse PCP-induced glutamate-to-GABA NTS. This result provides a new understanding of how clozapine, a pharmaceutical that is already used in humans, could potentially be employed to mitigate the cognitive deficits caused by PCP.

MATERIALS AND METHODS

Animals

All animal procedures were carried out in accordance with NIH guidelines and were approved by the University of California, San Diego, Institutional Animal Care and Use Committee. Mice were maintained on a 12:12-hr light/dark cycle (light on 7 am; light off 7 pm) with food and water *ad libitum*, at a temperature between 65 and 75 °F (18 and 23 °C) with 40–60% humidity.

VGLUT1-IRES-Cre mice (JAX#023527) (referred to as “VGLUT1-Cre” from here on) and Rosa26-LSL-H2B-mCherry (JAX#023139) (referred to as “mCherry” from here on), were obtained from Jackson Laboratories. Homozygous Rosa26-LSL-H2B-mCherry mice were bred with heterozygous VGLUT1-IRES-Cre animals and male offspring carrying both the Rosa26-LSL-H2B-mCherry and allele (i.e., VGLUT1-Cre +/-:: mCherry +/- mice) were used for experimental purposes.

Genotyping

All mice were routinely genotyped to identify the presence of the VGLUT1-IRES-Cre allele. No genotyping was instead needed to assess the presence of the Rosa26-LSL-H2B-mCherry allele since only mice homozygous for this allele were used as breeders. Tail tip samples were collected by tail biopsy and used for genomic DNA extraction. PCR was performed using Apex™ RED Taq DNA Polymerase Master Mix, with the primers and the genotyping protocol suggested by The Jackson Laboratory.

Pharmacological treatments

PCP administration*:*

Phencyclidine hydrochloride (PCP) was obtained from Sigma-Aldrich (Catalog # P3029) and dissolved in a sterile sodium chloride 0.9% solution (saline). Following an established protocol (Hashimoto *et al.,* 2005), adult mice (9-10 weeks of age) received daily subcutaneous injections of PCP (10 mg/kg), or saline (10 ml/kg) as control, for 10 days.

Clozapine administration*:*

Clozapine was obtained from Sigma-Aldrich (Catalog # C6305) and dissolved in DMSO to obtain a stock solution of 10 mg/ml. Right before use, the stock solution was further diluted in sterile saline to a final concentration of 0.5 mg/ml. As a control solution, we used DMSO dissolved 1:19 in saline (defined as Vehicle from now on). Three days after the end of PCP treatment, mice began receiving daily clozapine (5 mg/kg) or vehicle injections, for 2 additional weeks.

Immunohistochemistry

Mice were anesthetized using isoflurane and transcardially perfused with phosphate-buffered saline (PBS) and 4% paraformaldehyde (PFA). The brains were then post-fixed in 4% PFA for 12 h at 4°C. After fixation, brains were transferred to a solution of 30% sucrose in PBS at 4°C for 2 to 4 days. Brains were sectioned using a freezing microtome supplied with dry ice and 30 µm-thick coronal sections containing the PrL were collected.

For immunohistochemistry, sections were permeabilized and blocked for 2 h at room temperature (RT, 22-24°C) in blocking solution (5% normal horse serum, 0.3%

Triton X-100 in PBS) and then incubated overnight at 4°C with rabbit-anti-GABA primary antibody (Sigma-Aldrich, Catalog #A2052) diluted 1:1000 in blocking solution. The following day the primary antibody was removed, and the sections were washed 3 times for 15 minutes in 0.3% Triton X-100 in PBS before being incubated for 2h at RT in with secondary antibody Alexa Fluor-488 donkey-anti-rabbit (ImmunoResearch Labs, Catalog #705-545-003) diluted 1:500 in blocking solution. After 3 additional 15- minute washes in 0.3% Triton X-100 in PBS, sections were mounted with Fluoromount-G.

Imaging and data analysis

Images were acquired using a Leica SP5 confocal microscope with a 25x/0.95 water-immersion objective and a z resolution of 1 µm. The boundary of the PrL was determined based on the PrL cytoarchitecture, as previously described (Van De Werd and Uylings, 2014), and counts of the number of neurons co-expressing mCherry and GABA were performed on one of every six sections. The total number of co-expressing neurons was later calculated by multiplying the number of counted cells by 6. Counts were performed in a semi-automatic manner using the spot-function and the colocalize spot plugin in the Imaris-Bitplane software. Co-expression was then manually verified for each cell across all the z-stacks; only neurons showing colocalization in at least 3 consecutive images were included in the co-expression group.

Statistics

Data are expressed as mean +/- standard error of the mean (S.E.M.). Statistical analysis was performed on Graph-Pad Prism using one-way analysis of variance (Anova)

and Tukey's multiple comparison post hoc test. P-values less than 0.05 were considered statistically significant. The graphs were generated using Graph-Pad Prism.

RESULTS

We investigated if treatment with clozapine can reverse the PCP-induced glutamate-to-GABA neurotransmitter switch in prelimbic cortical neurons. VGLUT1-cre mice were crossed with an H2B-mCherry reporter line (**Fig. 3A**) to permanently label glutamatergic neurons even if they changed their neurotransmitter phenotype (**Fig. 3B**). This strategy allowed us to investigate whether, after drug-treatment, GABA starts to be expressed in neurons that were originally glutamatergic (i.e. mCherry+ cells) (**Fig. 3C**) as they show co-expression of mCherry and GABA (**Fig. 3D**).

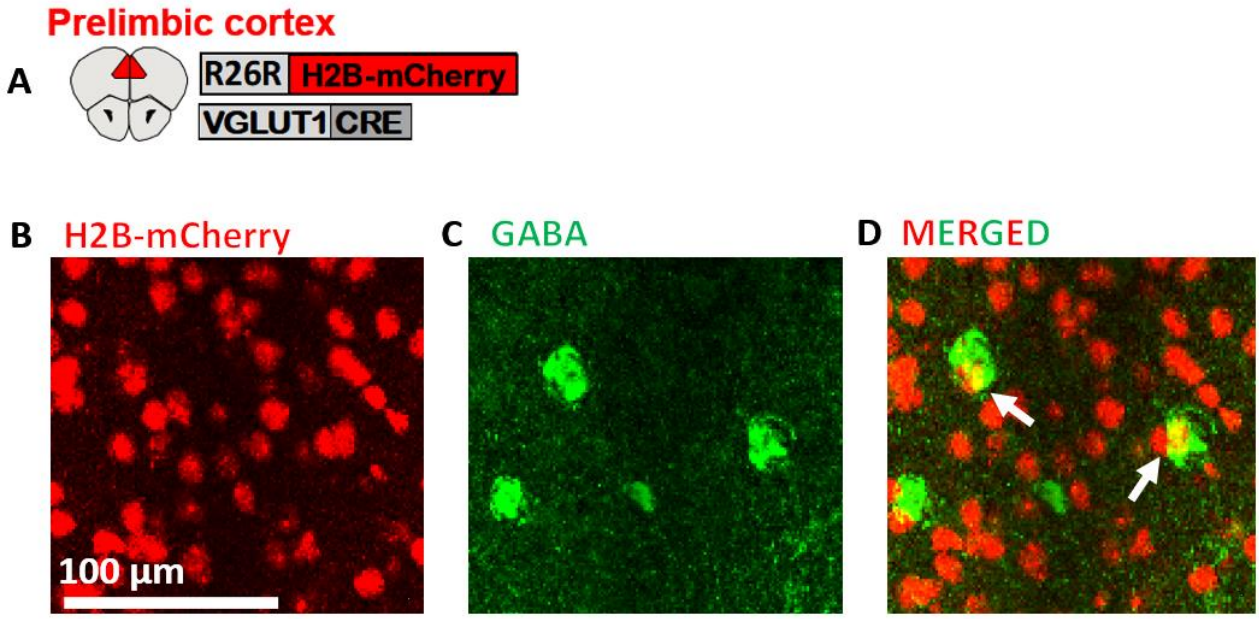

Figure 3: **Strategy to identify glutamate-to-GABA NTS in the prelimbic cortex. A**. Genetic mouse model used to permanently label glutamatergic neurons. **B**. Glutamatergic neurons highlighted by mCherry fluorescence in the prelimbic cortex of VGLUT1-cre+/-::mCherry+/- mice. **C.** GABA expression visualized through immunohistochemistry. **D.** Merging of the red and green channels shows colocalization of mCherry and GABA (arrows).

Mice were administered PCP (10 mg/kg) or saline for 10 days and received either clozapine (5 mg/kg) or vehicle for two additional weeks (**Fig. 4A**). Mice were then sacrificed, and coronal sections of the PrL were collected and immunostained for GABA.

Images were acquired using a confocal microscope and PrL boundaries were appropriately demarcated using ImageJ-Fiji (Materials and Methods).

In the prelimbic cortex of control mice (i.e. saline + vehicle) I counted an average of 578 glutamatergic neurons showing colocalization of GABA and mCherry signal (**Fig. 4B, 5**). This indicates that glutamatergic neurons co-expressing GABA are present in the PrL of naïve mice.

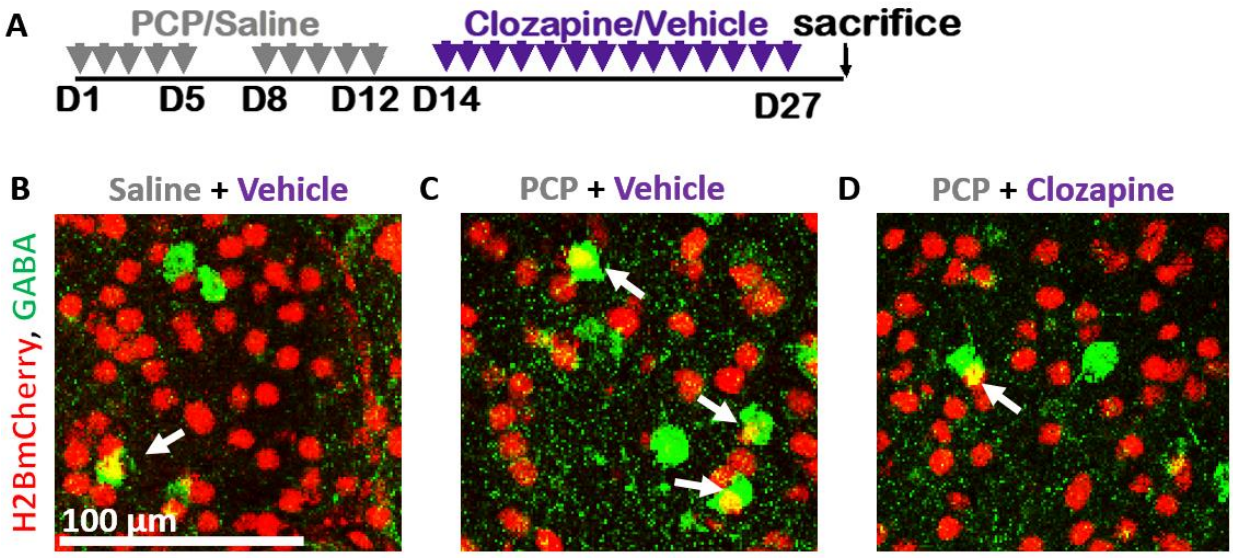

Figure 4: Images of colocalized mCherry+ and GABA+ cells across the three treatment groups. A. Timeline of the experimental procedure. **B.** Neurons co-expressing mCherry and GABA (arrow) are present in the prelimbic cortex of saline + vehicle control mice. **B.** The number of mCherry+/GABA+ co-expressing cells is increased in PCP + vehicle mice. **C.** The number of neurons co-expressing mCherry and GABA in mice that received clozapine after PCP treatment is comparable to that in saline + vehicle controls and less than that of mice that received PCP but not clozapine.

In line with previous data from our lab (Pratelli *et al.,* 2019), treatment with PCP significantly increases the number of neurons co-expressing GABA and mCherry to an average of 1104 (**Fig 4B, 5**). Statistical analysis using one-way Anova and Tukey's post-hoc test for multiple comparisons demonstrated the presence of a statistically significant difference in the number of mCherry+/GABA+ neurons between mice treated with PCP +

vehicle and saline + vehicle (P=0.0003). Analysis of the sections obtained from mice treated with PCP + clozapine showed that the number of mCherry+/GABA+ neurons was lower than in sections of mice treated with PCP + vehicle (**Fig. 4C**). Cell counts revealed that the number mCherry+/GABA+ neurons in PCP + clozapine treated mice (566 cells on average) was significantly decreased as compared to mice that received PCP + vehicle (one-way Anova, Tukey's multiple comparison test, P=0.0003), but not significantly different from saline + vehicle mice (one-way Anova, Tukey's multiple comparison test, P=0.9783; **Fig. 5**).

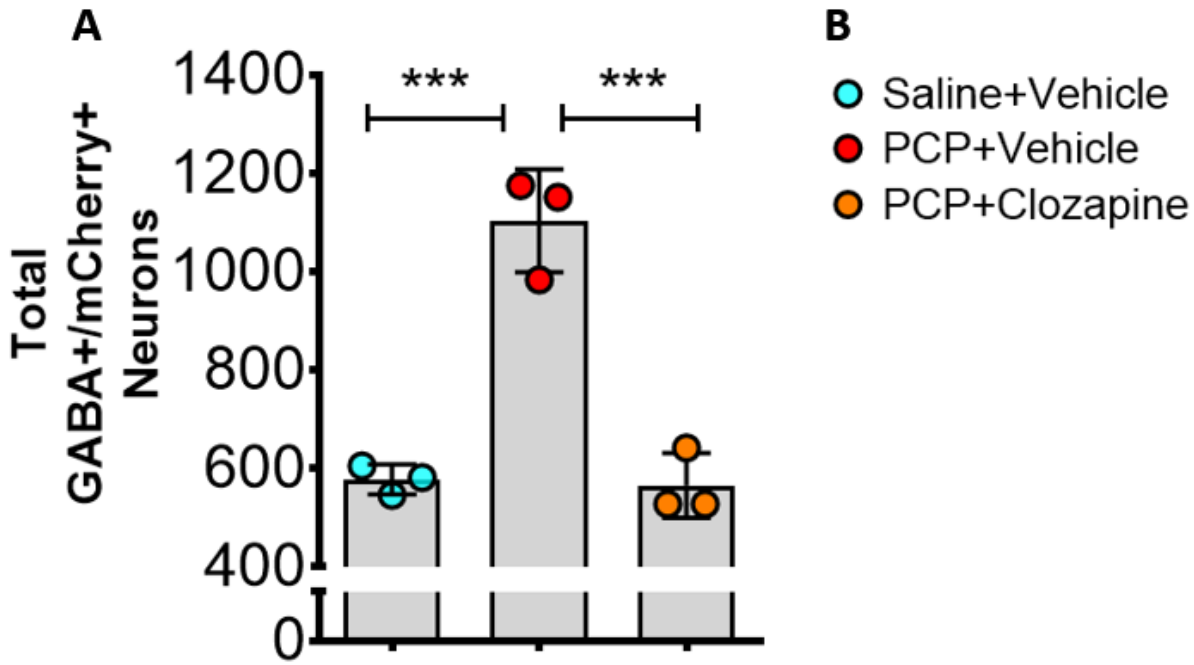

Figure 5: Number of GABA+/mCherry+ neurons in the prelimbic cortex of mice after treatment with PCP + clozapine. A) The graph shows the number of mCherry+/GABA+ cells in the prelimbic cortex of mice treated with saline + vehicle, PCP + vehicle or PCP + clozapine. Each dot represents the total number of cells counted in a mouse and the color indicates the treatment group. **B)** Treatment legend. Data are presented as mean +/- S.E.M. Statistical analysis was performed with one-way analysis of variance (Anova) and Tukey's multiple comparison post-hoc test. P-values less than 0.05 were considered statistically significant. Significance is expressed as *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$.

To verify that the differences in the number of mCherry+/GABA+ neurons that we observed across the 3 treatment groups were not due to uneven sampling of the PrL, I counted the total number of PrL mCherry+ cells (i.e., glutamatergic neurons). I obtained an average of 58068 mCherry+ neurons for the saline + vehicle group, of 58934 for the PCP + vehicle group and of 59856 for the PCP + clozapine group. First, I verified that these numbers correctly reflected the number of glutamatergic neurons in the PrL of adult mice (see Appendix). I then calculated the ratio of mCherry+/GABA+ neurons per 1000 mCherry+ cells (**Fig. 6**). As expected, the number of mCherry+/GABA+ neurons per 1000 mCherry+ cells in PCP + vehicle mice (average of 18/1000) was higher

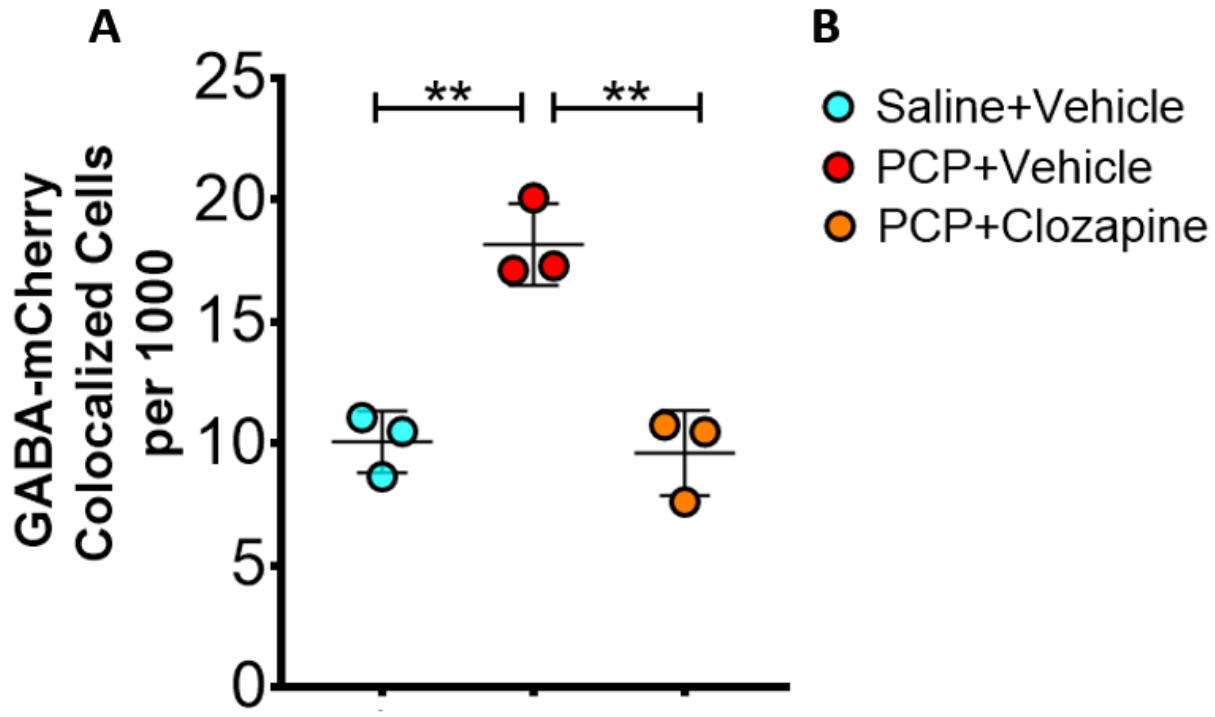

Figure 6: GABA+/mCherry+ neurons per 1000 mCherry+ cells in mice treated with PCP + clozapine, PCP + vehicle and saline + vehicle. A) The graph shows the number of GABA+/mCherry+ cells per-thousand mCherry+ glutamatergic neurons in mice treated with saline + vehicle, PCP + vehicle and PCP + clozapine (n=3). Each dot represents the total number of cells counted in a mouse and the color indicates the treatment group. **B)** Treatment legend. Data are presented as mean +/- S.E.M. Statistical analysis was performed with one-way analysis of variance (Anova) and Tukey's multiple comparison post-hoc test. Significance is expressed as *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$.

than in control mice (average of 10/1000; One- way Anova Tukey's multiple comparison test, P=0.0018) and in the PCP + clozapine group (average of 9/1000; one-way Anova Tukey's multiple comparison test, P=0.0014). Instead, no significant difference was detected between the number of co-expressing neurons between that saline + vehicle and PCP + clozapine groups (one-way Anova Tukey's multiple comparison test, P=0.9349; **Fig. 6**). Overall, these data indicate that clozapine successfully reverses the NTS induced by PCP.

Discussion

My results demonstrate that treatment with clozapine reverses the glutamate-to-GABA neurotransmitter switch induced by repeated injections of PCP. To identify switching cells, I counted the number of mCherry-labelled glutamatergic neurons co-expressing GABA in the PrL of VGLUT1-cre+/-::mCherry+/- mice treated with saline + vehicle, PCP + vehicle, or PCP + clozapine. I then compared the number of neurons co-expressing mCherry and GABA across different treatments. The number of co-expressing neurons was significantly reduced in mice that received both PCP and clozapine, as compared to animals treated only with PCP. To ensure that the differences in the number of co-expressing cells observed were not due to uneven sampling of the PrL, we also calculated the number of cells co-expressing GABA and mCherry per-thousand mCherry+ neurons. This confirmed the ability of clozapine to reverse the PCP-induced gain of GABA in prelimbic cortex glutamatergic neurons. Additional studies will be needed to consolidate the present findings. For example, the saline + clozapine control was not shown in this thesis since these data are still under analysis. However, this control group is important to exclude the possibility of clozapine itself causing the change in PrL neurotransmitter expression.

These results are consistent with our initial hypothesis. We hypothesized that, since clozapine has been documented to rescue PCP-induced cognitive deficits (Hashimoto *et al.,* 2005; Idris *et al.,* 2005) and preventing the NTS also prevents PCP-induced behavioral alterations (Pratelli *et al.,* 2020), clozapine can reverse PCP-induced NTS.

Clozapine's ability to reverse the switch can potentially be explained by the ability of this drug to rescue PCP-induced hyperactivity of PrL glutamatergic neurons (Kargieman *et al.,* 2007). Changes in brain activity have been repeatedly shown to modulate neurotransmitter specification and switching. The electrical activity of the surrounding neurons regulates neurotransmitter specification (Guemez-Gamboa *et al.,* 2014; Meng *et al.,* 2018). Furthermore, preliminary data from our lab show that chemogenetic manipulations of PrL activity can prevent and rescue the PCP-induced NTS (Pratelli *et al.,* 2021). Indeed, while acute PCP administration increases the activity of PrL pyramidal glutamatergic neurons (Kargieman *et al.,* 2007), when this change in activity is prevented through chemogenetic activation of local parvalbumin positive (PV) neurons, the switch is prevented (Pratelli *et al.,* 2021). The same strategy has also been used to reverse PCP-induced NTS after the end of PCP-treatment by subjecting mice to chemogenetic activation of PV inhibitory neurons daily for 10 days, beginning 2 days after the last PCP injection. It is therefore likely that clozapine's ability to rescue the switch is linked to its modulation of PrL activity. However, additional experiments will be needed to support this hypothesis. A first step will be, for example, to quantify the expression level of the immediate early gene and marker of activity c-fos in the PrL of mice acutely treated with PCP + vehicle, PCP + clozapine, saline + vehicle, or saline + clozapine, to observe if combined treatment with PCP + clozapine, decreases PCP-induced PrL hyperactivity.

Overall, we have shown that clozapine can be used to reverse a drug-induced glutamate-to-GABA switch in the PrL. However, further considerations are necessary when considering clozapine as a potential treatment for drug-induced behavioral alterations. Clozapine causes severe side effects and is generally used as a last resort

for treating severe psychiatric disorders such as schizophrenia and psychosis (De Berardis *et al.,* 2018). Therefore, other less invasive approaches to manipulate PrL activity and rescue the switch will need to be identified. At present, transcranial magnetic stimulation (Seibner *et al.,* 2009) appears to be a potential non-invasive strategy that may be used to manipulate PrL activity and interfere with NTS. However, practical limitations, mainly related to magnetic coil dimensions and precision of stimulation, make it difficult to test this treatment in mice.

In conclusion, this study has shed new light on the strategy and mechanisms that can be used to reverse PCP-induced NTS and likely rescue the associated cognitive deficits.

APPENDIX

To further validate our results, we wanted to make sure that the numbers of neurons we had quantified were in line with previously published data. For this validation we focused on the total number of mCherry-positive glutamatergic neurons counted: 58068 (saline + vehicle group), 58934 (PCP + vehicle), and 59856 (PCP + clozapine). For reference we used the data of Keller *et al.,* 2018 reporting that the total density of neurons in the frontal cortex is 121,000 $\pm$ 52,000 per mm^3. We then used the Paxinos Allen Brain Atlas to estimate the volume of the PrL. The shape of the PrL is a triangular prism, with the X= ~ 0.96 mm, Y= ~ 1.4 mm, Z= ~ 1.3 mm, despite the height shrinkage moving along the rostro-caudal axes. We extrapolate a volume of ~ 0.6 mm^3 to account for this shrinkage, even if changes of the shape across the rostro-caudal axes make it difficult to have a precise quantification. Therefore, if the density of neurons in the region is 121,000 per mm^3, the total number of cells in the PrL is ~ 72600 (121,000 cells/mm^3 x 0.6 mm^3). While this number includes both GABAergic and glutamatergic cells, we know that the percentage of glutamatergic cells in the PFC is ~80% of the total (Santana and Artigas, 2017). We therefore calculated a total number of ~58080 PrL glutamatergic neurons, which is in line with the results of our counts (a total of 58068 (saline + vehicle group), 58934 (PCP + vehicle), and 59856 (PCP + clozapine).

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