Prior Antipsychotic Drug Treatment Prevents Response to Novel Antipsychotic Agent in the Methylazoxymethanol Acetate Model of Schizophrenia

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Trials of novel compounds for the treatment of schizophrenia are typically tested in patients following brief withdrawal of ongoing medication despite known long-term changes in the dopamine (DA) system following chronic antipsychotic drug therapy. The present study explored the impact of withdrawal from repeated haloperidol (HAL) treatment, as well as the response to a novel α5 gamma-aminobutyric acid (GABA A) receptor positive allosteric modulator (α5PAM), on the activity of the DA system in the methylazoxymethanol acetate (MAM) neurodevelopmental model of schizophrenia. Electrophysiological recordings were conducted from DA neurons in the ventral tegmental area of MAM and saline (SAL) rats following 7-day withdrawal from repeated HAL (21 d, 0.6 mg/kg, orally). In separate animals, amphetamine-induced locomotion was measured to assess changes in DA behavioral sensitivity. SAL rats withdrawn from HAL demonstrated reduced spontaneous DA neuron activity along with an enhanced locomotor response to amphetamine, indicative of the development of DA supersensitivity. Both α5PAM treatment and ventral hippocampal (vHPC) inactivation reversed the DA neuron depolarization block following HAL withdrawal in SAL rats. In contrast, MAM rats withdrawn from HAL exhibited reduced spontaneous DA activity and enhanced locomotor response to amphetamine compared with untreated SAL rats; however, this condition was unresponsive to α5PAM treatment or vHPC inactivation. Withdrawal from prior HAL treatment interferes with the therapeutic actions of this novel treatment in the MAM model of schizophrenia. Consequently, testing novel compounds on chronically treated schizophrenia patients may be ineffective.

Keywords: haloperidol/ventral tegmental area/withdrawal/schizophrenia/GABA receptor positive allosteric modulator/dopamine supersensitivity

Introduction

Currently, between 49%–74% of schizophrenia patients discontinue antipsychotic drug (APD) treatment due to adverse side effects and limited efficacy.1,2 APDs target the pathological increase in dopamine (DA) activity3,4; however, recent studies suggest that disruptions in the DA system are probably secondary to other pathologies in schizophrenia. This has driven a search for novel non-DA agents to treat schizophrenia. However, promising novel therapeutics in preclinical studies typically fail to demonstrate efficacy in clinical trials. At issue is the testing of novel compounds in patients exposed to APDs for years and briefly withdrawn.5–8 How prior APD exposure and subsequent withdrawal affects the response of the DA system has not been adequately assessed. Withdrawal from repeated APD treatment increases DA D2 receptor expression and sensitivity in caudate putamen and nucleus accumbens9–13 and probably contributes to DA supersensitivity psychosis observed in a subset of treatment-resistant schizophrenia patients.14–16

Another major limitation of preclinical studies on the efficacy of APDs is in the use of normal animals. Utilizing a well-established animal model of schizophrenia would be more suitable. The methylazoxymethanol acetate (MAM) model involves the administration of a DNA-methylating agent to pregnant dams on gestational day (GD) 17 to interfere with normal neural development, which yields offspring demonstrating both structural and behavioral abnormalities consistent with those observed in schizophrenia patients.17–20 Previous work with the MAM model attributed the underlying DA pathology to aberrant activation of the ventral hippocampus (vHPC)19; moreover, these animals demonstrate a response to first- and second-generation APDs more consistent with that seen clinically.21 Treatment with a novel α5 gamma-aminobutyric acid (α5GABA A) receptor positive allosteric
modulator (α5PAM) in MAM rats reduces DA activity by directly treating the aberrant vHPC output.22

The present study explored the consequence of haloperidol (HAL) withdrawal on DA system activity, as well as the behavioral response to amphetamine, in MAM-treated rats, and its impact on the efficacy of subsequent treatment with a novel α5PAM.

Methods

Experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh. Experiments utilized adult male offspring of pregnant dams treated with saline or MAM. Animals were housed in a temperature (22°C)- and humidity (47%)-controlled environment (12-h light/dark cycle; lights on at 7 AM) with ad libitum access to food and water. For behavioral experiments, animals were housed under a reverse light cycle (lights on at 7 PM) and tested during the lights-off period.

Drug Preparation and Administration

For detailed methods pertaining to drug preparation and administration, refer to the supplementary data.

Methylazoxymethanol. MAM administration was performed as described previously.19,20,22,23 Briefly, timed pregnant female Sprague–Dawley rats (Hilltop) were administered MAM (20 mg/kg, IP) or saline (1 ml/kg, IP) on GD 17. Multiple litters of male offspring (aged 3–4 mo) were used.

Haloperidol. Animals were individually housed to facilitate daily HAL administration. HAL (Sigma-Aldrich; 0.6 mg/kg in 0.1M glacial acetic acid, pH = 5.5) was administered in the home cage (21 d, orally). Control saline (SAL) and MAM rats received vehicle (VEH)-treated wafers (21 d). Similar HAL treatment induces depolarization block of DA neurons in substantia nigra24 and ventral tegmental area (VTA),25,26 as well as in reduced striatal DA release.24,27 All behavioral and physiological experiments occurred 7 days after the last HAL or VEH treatment.

Gamma-aminobutyric Acid Positive Allosteric Modulator. SH-053-2′F-R-CH₃ (α5PAM) is a novel α5PAM. Detailed methods describing the synthesis of SH-053-2′F-R-CH₃ have been provided previously.28 For electrophysiological recordings, animals were administered VEH (2 ml/kg, IV) or SH-053-2′F-R-CH₃ (0.1 mg/kg, IV) > 20 minutes prior to DA neuron sampling. For behavioral experiments, VEH (4 ml/kg, IP) or SH-053-2′F-R-CH₃ (10 mg/kg, IP) was administered 1 hour prior to testing.

Ventral HPC Inactivation. Tetrodotoxin (Sigma-Aldrich; TTX; 1 µM/0.5 µl) or Dulbecco’s phosphate-buffered saline (0.5 µl; dPBS) was infused into the vHPC (figure 1; anterior-posterior (AP), –5.6 mm; mediolateral (ML), +5.0 mm from bregma, and −4.5 mm ventral to brain surface) prior to electrophysiological recordings.

![Fig. 1. Schematic representation of cannula sites in ventral hippocampus with representative tissue (modified from Paxinos and Watson).35](image-url)
Following infusion, the cannula was left in place for 2 minutes prior to removal.

**Apomorphine.** After electrophysiologically sampling the VTA, apomorphine (Sigma-Aldrich; 80 or 120 μg/kg in saline, IV) was administered 30 minutes prior to resampling the VTA, commencing 0.1 mm posterior to the last track made prior to apomorphine.

**Electrophysiological Recordings and DA Neuron Classification**

Animals were anesthetized with chloral hydrate (Sigma, 400 mg/kg, IP) and supplemented periodically (IV) to maintain suppression of the hind limb withdrawal reflex. Body temperature was maintained at 37°C with a temperature-controlled heating pad (Fintronics). Single glass microelectrodes (WPI; impedance 6–8 MΩ) filled with a 2% Chicago Sky Blue solution (2M NaCl; Sigma) were used to sample spontaneous neural activity in the VTA using well-established techniques. The activity of each DA neuron was recorded for 5 minutes. Three parameters were measured: (1) population activity (number of spontaneously active DA neurons per electrode track), (2) basal firing rate, and (3) the proportion of action potentials occurring in bursts (commencing 2 spikes with an interspike interval of <80 ms, and terminating when interspike interval >160 ms).

**Amphetamine-Induced Locomotor Activity**

After 7 days withdrawal from repeated HAL, the SAL and MAM rats were administered α5PAM (10 mg/kg, IP) or VEH (4 ml/kg, IP) in their home cages and transported to the behavioral testing room. Testing commenced 1 hour after α5PAM or VEH injection. Spontaneous locomotor activity for baseline (30 min) and postamphetamine injection (90 min; 0.5 mg/kg, IP) periods was measured in an open field arena by beam breaks in the x–y plane and recorded with TruScan software (Coulbourn Instruments, Allentown, PA). Total distance travelled was computed for each 5-minute epoch.

**Histology**

Following electrophysiological experiments, rats were killed with an overdose of chloral hydrate (additional 400 mg/kg, IV) and the recording location was marked electrophoretically (Kation Scientific; −13 μA constant current, 30 min). Following decapitation, brains were removed, fixed (8% wt/vol paraformaldehyde in PBS), and cryoprotected (25% wt/vol sucrose in PBS) until saturated. Brains were sectioned (60-μm coronal sections), mounted onto gelatin-chrom alum-coated slides, and stained with a mixture of cresyl violet and neutral red for histochemical verification of electrode sites.

**Statistics**

Electrophysiological analysis was performed using the computer software PowerLab (AD Instruments) and Nex (NEX Technologies). Locomotor behavior was recorded using TruScan software (Coulbourn Instruments). All data are represented as the mean ± standard error of the mean. Unless otherwise stated, electrophysiological data were assessed with 2-way ANOVA (SigmaPlot) while behavioral data were tested with 2-way repeated-measures ANOVAs. Holm-Sidak post hoc comparisons were performed after a significance of $P < .05$.

**Results**

**HAL Pretreatment Disparately Affects VTA DA Neurons in SAL and MAM Rats**

We reported previously that MAM rats exhibit an increase in VTA DA neuron population activity that can be reversed by treatment with a novel α5PAM, SH-053-2F-R-CH₃. Here, we explored how this response was altered following repeated HAL treatment.

Electrophysiological recordings were conducted in 27 SAL and 27 MAM rats divided among untreated controls, VEH-treated controls, rats withdrawn from repeated HAL + VEH (2 ml/kg, IV) and rats withdrawn from repeated HAL + α5PAM (0.1 mg/kg, IV). A total of 159 DA neurons were recorded from SAL rats and 253 neurons were recorded from MAM rats. Data from the untreated and VEH-treated controls were not significantly different, and subsequent statistical comparisons used the combined data from both groups.

Untreated MAM rats demonstrated the anticipated elevation in DA population activity compared with SAL rats (figure 2A; 2-way ANOVA main effects: for saline, $F = 58.98, P < .000001$; for α5PAM, $F = 32.68, P < .000001$; MAM-by-α5PAM interaction: $F = 11.29, P = .000026$; post hoc MAM control vs SAL control: $t = 5.84, P = .000007$). In both untreated SAL and MAM rats, α5PAM treatment significantly reduced the number of active DA neurons ($t = 3.70, P = .00062$ and $t = 7.04, P < .000001$, respectively). In SAL rats, following HAL withdrawal, there was also a significant decrease in the number of active DA neurons per track compared with that in untreated SAL animals ($t = 4.20, P = .00014$), which was reversed by α5PAM treatment ($t = 1.38,$...
In MAM rats, HAL withdrawal also resulted in a significant reduction in the number of spontaneously active DA neurons ($t = 7.64, P < .0000001$); however, α5PAM treatment had no additional effect on the reduction in DA activity ($t = 7.88, P < .0000001$). This is in direct contrast with both our previous reports and current data in control rats that had not been treated with HAL, showing that α5PAM treatment restores normal DA activity in drug-naïve MAM rats. Notably, withdrawn MAM rats continued to demonstrate elevated population activity compared with withdrawn SAL rats ($t = 2.40, P = .021$), suggesting a persistent pathological increase in DA activity in HAL-withdrawn MAM rats despite prior HAL treatment. Consequently, withdrawal from HAL treatment appears to have a differential impact on therapeutic efficacy of α5PAM administration in SAL and MAM rats.

DA neurons in SAL rats withdrawn from repeated HAL and treated with α5PAM were firing significantly faster compared with untreated SAL rats (figure 2B; 2-way ANOVA interaction between MAM and α5PAM treatment: $F = 3.22, P = .02$; post hoc SAL control vs HAL withdrawn SAL + α5PAM: $t = 2.68, P = .0075$). This pattern of activity is consistent with the removal of depolarization block reported in earlier studies. Withdrawal from HAL ($t = 0.085, P = .93$) and α5PAM treatment in untreated controls or following HAL withdrawal ($t = 0.30, P = .76$ and $t = 1.29, P = .19$, respectively) had no effect on the firing rate of DA neurons recorded in MAM rats.

No significant differences were found in the percentage of spikes occurring in bursts for DA neurons recorded in SAL and MAM rats ($* P < 0.05$).

**HAL Pretreatment Differentially Affects VTA DA Neuron Response to vHPC Inactivation in SAL and MAM Rats**

Previously, we showed that inactivation of the vHPC with TTX restored VTA DA neuron activity to control levels in MAM rats. In this study, we examined whether the elevated DA neuron activity in HAL-withdrawn MAM rats was also sensitive to vHPC inactivation.

Electrophysiological recordings were conducted in 18 SAL and 18 MAM rats divided equally among untreated controls, rats withdrawn from repeated HAL + dPBS infused into the vHPC (0.5 μl), and rats withdrawn from repeated HAL + TTX infused into the vHPC (1 μM/0.5 μl). The same groups of untreated SAL and MAM rats reported above with the α5PAM results served as controls for this experiment. A total of 108 DA neurons were recorded from SAL rats and 169 neurons were recorded from MAM rats.

HAL-withdrawn SAL rats continued to exhibit a significant decrease in the number of active DA neurons per track compared with untreated SAL animals (figure 3A; 2-way ANOVA main effects for MAM: $F = 26.69, P = .000016$, and for α5PAM: $F = 47.44, P < .0000001$, as well...
as significant interaction between MAM and α5PAM treatment: $F = 7.97, P = .0017$; post hoc SAL control vs HAL-withdrawn SAL: $t = 4.45, P = .00011$). However, in contrast with the lack of effect of vHPC inactivation in untreated control rats,29,36 inactivating the vHPC reversed the withdrawal-induced decrease in spontaneously active DA neurons in SAL rats ($t = 1.93, P = .06$), suggesting altered regulation of DA activity by the vHPC in SAL rats withdrawn from HAL. In contrast, inactivating the vHPC in MAM rats did not affect the decrease in VTA DA neuron activity observed following HAL withdrawal. Both VEH ($t = 9.11, P < .000001$) and TTX infusions in vHPC in MAM rats following HAL withdrawal ($t = 7.20, P < .000001$) maintained a significant reduction in DA neuron spontaneous activity compared with drug-naive MAM rats, suggesting that the decrease was due to withdrawal from HAL. As reported earlier, withdrawn MAM treated rats continued to demonstrate elevated population activity compared with withdrawn SAL rats ($t = 2.38, P = .04$). Consequently, inactivating the vHPC was ineffective in further reducing the level of DA activity in withdrawn HAL-treated MAM rats.

There were no significant differences in the average firing rate (MAM: $F = 1.86, P = .17$; or TTX: $F = 0.90, P = .40$; figure 3B) or burst firing (MAM: $F = 0.26, P = .61$; or TTX: $F = 2.39, P = .09$; figure 3C).

Failure to Reverse Depolarization Block in MAM Rats Following HAL Withdrawal

We reported previously that repeated HAL induced DA neuron hyperactivation-driven depolarization block.24-26 Activity was restored in these neurons by inhibition via administration of a low dose of apomorphine (80–120 μg/kg, IV).21,24,38 In a subset of SAL (n = 3) and MAM (n = 6) rats withdrawn from HAL, VTA activity was sampled before and after administration of apomorphine. In HAL-withdrawn SAL rats, administration of apomorphine significantly increased the number of DA neurons firing compared with the pre–apomorphine sampling period (figure 4; 2-way ANOVA interaction between the pre- and postapomorphine time points and MAM treatment: $F = 7.96, P = .01$; $t = 3.26, P = .0068$). In contrast, in HAL-withdrawn MAM rats, apomorphine did not affect the number of DA neurons firing ($t = 0.0062, P = .99$). DA neurons recorded following apomorphine exhibited slower firing rates than those recorded preinjection for both SAL and MAM rats (supplementary figure 1). This would suggest that apomorphine was effective in removing depolarization block in SAL, but not in MAM, animals following HAL withdrawal.

Reversal of Amphetamine Hyperresponsivity by α5PAM Is Disrupted in MAM Rats Following HAL Withdrawal

We showed previously that MAM rats exhibit a heightened locomotor response to psychostimulants, which parallels the elevation in DA neuron activity.19,22 α5PAM administration reversed both DA neuron hyperactivity and the increased locomotor response to amphetamine in MAM-treated rats.19,22 In normal animals, the enhanced behavioral response to amphetamine following withdrawal from repeated HAL was attributed to DA system
supersensitivity. Here, we examined whether the alteration in DA system activity following withdrawal from repeated HAL produced an equivalent change in the behavioral response to amphetamine in MAM rats.

Behavioral experiments were conducted in 32 SAL and 32 MAM rats divided among VEH-treated wafer controls + VEH (4 ml/kg, IP), VEH-treated wafer controls + α5PAM (10 mg/kg, IP), rats withdrawn from repeated HAL + VEH (4 ml/kg, IP), and rats withdrawn from repeated HAL + α5PAM (10 mg/kg, IP). Total distance traveled was assessed for the 30-minute baseline (5-min BIN) and 90-minute postamphetamine testing periods. The behavioral data were analyzed with repeated-measures ANOVA, and subsequent post hoc comparisons were made between groups within the individual 5-minute bins. VEH-treated MAM rats, SAL rats withdrawn from repeated HAL, and SAL rats administered the α5PAM displayed enhanced activity when first placed in the locomotor chamber (supplementary figure 2A). However, all groups displayed similar levels of activity by the end of the baseline period (supplementary figure 2B).

Consistent with previous reports, VEH-treated MAM rats demonstrated a significantly larger peak locomotor response to amphetamine than VEH-treated SAL rats (supplementary figure 3). Following amphetamine injection, SAL rats withdrawn from repeated HAL showed an elevated locomotor response compared with VEH-treated SAL rats (figure 5a; repeated-measures ANOVA main effect of treatment: F = 3.73, P = .02; and treatment-by-BIN interaction effect: F = 2.57, P < .0000001; time point comparisons: 15 min: t = 2.96, P = .0034; 25 min: t = 2.48, P = .013; 40 min: t = 2.46, P = .014; and 60 min: t = 2.62, P = .0093). In contrast, administration of α5PAM in SAL rats withdrawn from repeated HAL caused a significant reduction in locomotor activity compared with VEH-treated SAL rats (time point comparisons: 5 min: t = 3.04, P = .0026; and 10 min: t = 3.88, P = .00013). These data are consistent with previous reports of enhanced behavioral responses to psychostimulants following withdrawal from HAL.

For the first 5 minutes postamphetamine treatment, MAM rats withdrawn from repeated HAL had a significantly lower locomotor response compared with VEH-treated MAM rats (figure 5b; repeated-measures ANOVA main effect of treatment: F = 3.68, P = .024; and treatment-by-BIN interaction effect: F = 2.14, P = .000012; 5 min: t = 3.21, P = .0019). As shown previously, α5PAM treatment significantly reduced the locomotor response to amphetamine in MAM rats compared with that in controls (time points: 5 min: t = 3.44, P = .0091; 10 min: t = 5.69, P = .000002; 15 min: t = 5.13, P = .000018; 20 min: t = 3.28, P = .0015; and 25 min: t = 3.55,

Fig. 4. Administration of apomorphine increased the number of spontaneously active dopamine (DA) neurons in saline (SAL) rats withdrawn from repeated haloperidol (HAL) treatment, presumably by reversal of DA neuron depolarization block, while having no effect on the number of active DA neurons in HAL-withdrawn methylazoxymethanol acetate (MAM) rats (*P < 0.05).

Fig. 5. Repeated haloperidol (HAL) treatment enhanced the locomotor response to D-amphetamine in saline (SAL) animals, (A) which was reduced by pretreatment with the α5 gamma-aminobutyric acid (GABA), receptor positive allosteric modulator (α5PAM). Methylazoxymethanol acetate (MAM) rats treated repeatedly with HAL exhibited a locomotor response following D-amphetamine similar to untreated MAM rats. (B) However, repeated HAL treatment blocks the effect of α5PAM pretreatment in decreasing the locomotor response in MAM rats (*P < 0.05).
Indeed, comparing the efficacy of APDs is complicated by clinical trials beset with high attrition rates and inadequate reduction in rehospitalization.\textsuperscript{1,40–43} The excessive activation of the DA system in the MAM model is probably the result of overdrive from the vHPC.\textsuperscript{19} Previous work has identified a potential novel therapeutic, a \(\alpha_5\)PAM, that targets GABA\(_A\), \(\alpha_5\) receptor subunits localized to the hippocampus to reduce this overactivity.\textsuperscript{44–51} Previously, administration of the \(\alpha_5\)PAM was effective in restoring DA neuron activity and the behavioral response to amphetamine to control levels in MAM rats.\textsuperscript{22} Here we show that MAM rats continued to exhibit greater activation of the DA system compared with SAL rats, demonstrating that \(\alpha_5\)PAM treatment was no longer effective in reducing DA system activity. In contrast, \(\alpha_5\)PAM treatment in HAL-withdrawn SAL rats instead increased DA neuron spontaneous activity.

This lack of response to the \(\alpha_5\)PAM appears to be due to a disconnect between vHPC overactivity and DA neuron firing. Indeed, directly inactivating the vHPC with TTX failed to reverse the elevated DA neuron activity following HAL withdrawal, in contrast with that in HAL-withdrawn SAL rats, in which both the \(\alpha_5\)PAM and vHPC inactivation restored normal DA system activity presumably by decreasing DA neuron overdrive. This suggests that alterations resulting from HAL withdrawal in an alternative brain region, such as nucleus accumbens, probably accounts for the changes in DA system activity. Although it is possible that these results may not extend to all first- and second-generation APDs, there are vital clues for the inconsistencies between preclinical trials for novel therapeutics that utilize normal subjects and subsequent clinical trials with patients.

**DA Receptor Supersensitivity Following Repeated Antipsychotic Treatment**

In rats, repeated blockade of DA D2 receptors increases D2 receptor expression and binding in both the nucleus accumbens and caudate putamen.\textsuperscript{52–55} DA supersensitivity following APD withdrawal in animals, and the reemergence of psychotic symptoms in patients, is associated with increased D2 receptor high-affinity state.\textsuperscript{12,56} In animal studies, DA supersensitivity is associated with an enhanced response to psychostimulants,\textsuperscript{12,57} as observed here. Therefore, following APD treatment, there is an alteration in the DA system, which could contribute to psychosis independent of the state of the vHPC system.

**Depolarization Block Following Repeated HAL**

Previous studies have shown a decrease in spontaneous DA activity immediately after 21 days of HAL treatment in normal rats,\textsuperscript{21,24,25} which is reversed by the administration of apomorphine.\textsuperscript{21,38} The failure of apomorphine in the case of MAM, but not in SAL, rats withdrawn from repeated HAL would suggest that the decrease observed...
is not due to depolarization block. This conflicts with the reduced dopamine activity observed in MAM rats immediately after 21 days of HAL treatment that is reversed by apomorphine. In addition, the finding of vHPC inactivation reversing depolarization block in SAL rats withdrawn from repeated HAL suggests that hippocampal drive is additive with the D2 blockade–mediated striatal depolarization block. Whereas hippocampal overdrive with N-methyl-D-aspartate or bicuculline fails to drive depolarization block, whereas hippocampal overdrive with N-methyl-D-aspartate or bicuculline fails to drive depolarization block. Overall, these data support a model whereby changes to the dopamine system occurring during the withdrawal period in the MAM, but not in SAL, rats alter the depolarization block of these neurons. It remains untested whether there are distinct physiological consequences, in either the striatum or the vHPC, to withdrawal from repeated HAL between SAL and MAM animals.

Conclusion

This study suggests that brief withdrawal from repeated APD changes the system such that novel agents may no longer be effective—ie, by inducing DA supersensitivity, the long-term APD treatment has “addicted” the system to D2-blocking agents. This could account for the failure of novel drugs in clinical trials on schizophrenia patients with a history of APD treatment.

Supplementary Material

Supplementary material is available at http://schizophreniabulletin.oxfordjournals.org.

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