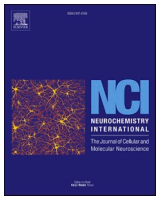
Neurochemistry International 178 (2024) 105785

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/neuint



Novel psychoplastogen DM506 reduces cue-induced heroin-seeking and inhibits tonic GABA currents in the Prelimbic Cortex

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ARTICLE INFO

Keywords:
DM506
Psychoplastogens
Heroin
Tonic GABA
Cue-induced seeking
Rats

ABSTRACT

Opioid use disorder is a major public health crisis that is manifested by persistent drug-seeking behavior and high relapse frequency. Most of the available treatments rely on targeting opioid receptors using small molecules that do not provide sustained symptom alleviation. Psychoplastogens are a novel class of non-opioid drugs that produce rapid and sustained effects on neuronal plasticity, intended to produce therapeutic benefits. Ibogalogs are synthetic derivatives of iboga alkaloids that lack hallucinogenic or adverse side effects. In the current study, we examine the therapeutic potential of DM506, a novel ibogalog lacking any cardiotoxic or hallucinogenic effects, in cue-induced seeking behavior following heroin self-administration. At a single systemic dose of 40 mg/kg, DM506 significantly decreased cue-induced seeking in both male and female rats at abstinence day 1 (AD1) following heroin self-administration. Upon re-testing for cue-induced seeking at AD14, we found that males receiving DM506 continued to show decreased cue-induced seeking, an effect not observed in females. Since there is evidence of psychedelics influencing tonic GABA currents, and opioid and psychoplastogen-mediated neuroadaptations in the medial prefrontal cortex (PrL) underlying its functional effects, we performed patch-clamp recordings on PrL slices of drug-naïve rats with an acute application or chronic incubation with DM506. Tonic GABA current was decreased in slices incubated with DM506 for 2 h. qPCR analysis did not reveal any differences in the *mRNA* levels of $GABA_A$ receptor α and δ subunits at AD14 in heroin and saline self-administered animals that received vehicle or DM506 at AD1. Overall, our data indicate that DM506 attenuates cue-induced heroin seeking and inhibits tonic GABA current in the prelimbic cortex.

1. Introduction

Opioid use disorder (OUD) is a chronic relapsing psychiatric disease that has reached epidemic proportions in the United States (Dydyk et al., 2024). OUD is characterized by a profound withdrawal syndrome and high rates of relapse. According to estimates from the Centers for Disease Control and Prevention, $78.5 billion was spent in 2016 on opioid-related healthcare costs, lost productivity, treatments, and law and enforcement (Rudd et al., 2016). Currently, available pharmacotherapies primarily target μ-opioid receptor opioid receptors (MOR) and are referred to as medications for opioid use disorder (MOUDs). First-line treatments such as methadone (MOR agonist), buprenorphine (MOR agonist), can cause toxicity and, in some cases, have abuse liability (Maxwell and McCance-Katz, 2010). Other drugs such as clonidine, which is used off-label to treat opioid withdrawal and is in the same drug class as the FDA-approved opioid withdrawal medication

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https://doi.org/10.1016/j.neuint.2024.105785
Received 4 April 2024; Received in revised form 10 May 2024; Accepted 1 June 2024
Available online 3 June 2024

lofexidine (Lucemyra), have adverse effects that include cardiac aberrations, CNS depression, respiratory distress, and hypotension to mention a few (Toce et al., 2018). Thus, there is a pressing need to develop improved therapies that rely on alternative mechanisms targeting biological pathways other than the MORs.

Psychoplastogens belong to a new class of therapeutic compounds with robust abilities to produce a rapid measurable change in neuronal structure and function that can be long-lasting (Vargas et al., 2021). They are known to modulate levels of neurotransmitters like dopamine (DA), serotonin (5-HT), glutamate, and γ-aminobutyric acid (GABA) in brain regions such as the thalamus and prefrontal cortex (Nichols, 2016; Wojtas et al., 2022). Compounds that qualify to be psychoplastogens often produce measurable changes in neuronal plasticity within a short period following a single administration. Psychoplastogens trigger a labile state to neuronal plasticity to facilitate the reshaping of neuronal circuits that are often long-lasting (Olson, 2018). Such persistent effects on neural substrates have made psychoplastogens desirable drug candidates for treating diseases like depression and post-traumatic stress disorder (PTSD). The second-generation psychoplastogens are non-hallucinogenic and non-toxic, circumventing many, if not all, of the potential drawbacks presented by their hallucinogenic counterparts (Vargas et al., 2021). This has presented the opportunity to examine the effects of these second-generation psychoplastogens on animal models of human diseases. The anti-addictive effects of second-generation psychoplastogens have not been extensively explored in relation to OUD.

"Ibogalogs" are a novel family of three-ringed compounds derived from the structural scaffold of iboga alkaloids. This unique structural identity imparts non-hallucinogenic and non-toxic pharmacological profiles, making ibogalogs potential drug candidates for therapeutic use (Cameron et al., 2021). The pharmacological effect of ibogalogs in animal models of substance use disorder has been sparsely explored. Ibogaine has been shown to reduce operant self-administration of ethanol in a rat model (He et al., 2005). The only ibogalogs that have been studied so far using rodent models of contingent and non-contingent opioid administration are tabernanthalog (TBG) and Ibogaine (Cameron et al., 2021; Glick et al., 1997; Heinsbroek et al., 2023; Pearl et al., 1995). 3-Methyl-1,2,3,4,5,6-hexahydroazepino[4,5-b]indole fumarate (DM506- Fig. 1) is a novel ibogalog that lacks any hallucinogenic, cardiac, or tremorgenic properties often seen in the parent compounds (Arias et al., 2024). Interestingly, DM506 has shown anxiolytic-, antidepressant-like, and anti-neuropathic activity in rodents (Arias et al., 2024; submitted manuscript). However, the anti-addictive effect of DM506 remains to be determined. We examined the acute and long-term pharmacological effect of DM506 using a contingent model of heroin self-administration where rats exhibit escalated cue-induced seeking at prolonged when compared to early abstinence time points (Kuntz et al., 2008; Kuntz et al., 2008).

Neuronal function and behavioral control are critically regulated by inhibitory neurotransmission (Centanni et al., 2017). Intriguingly, abstinence from chronic morphine exposure in a non-contingent model induces inhibitory long-term depression in the hippocampus (Han et al., 2015) indicating its involvement in opioid-related neuroplasticity. DM506 has a complex pharmacological profile including nicotinic and serotonergic actions, however, they have not been tested for GABAergic functions in-vivo. Since both nicotinic and serotonergic systems affect extracellular levels of GABA in the prefrontal cortex (PFC) (Abi-Saab, 1999; Aracri et al., 2010), we further investigated the role of DM506 in modulating tonic GABA currents in the prelimbic cortex (PrL). Among various $GABA_A$ receptor subunits expressed in the CNS, α4, α5, α6, and δ are located extrasynaptically (Wu and Sun, 2015) and are known to influence tonic currents. Additionally, these receptor subunits are expressed in cortical regions (Agrawal and Dwivedi, 2020; Wearne et al., 2016). Thus, we examined the *mRNA* expression of α&δ GABA subunits from the PrL region of rats that underwent DM506 or vehicle injections at abstinence day 14 (AD14) following saline or heroin self-administration (SA).

2. Methods

2.1. Animals

31 male (250–275 g) and 32 female (200–225 g) Sprague–Dawley rats (HillTop Lab Animals Inc, Scottdale, PA) were age-matched and used for all behavioral and biochemical experiments (at Marshall University). Wild type Long Evan rats from a CX3CR1-cre rat breeding colony (original male breeder purchased from Rat Resource and Research Center and females purchased from Charles River) were used

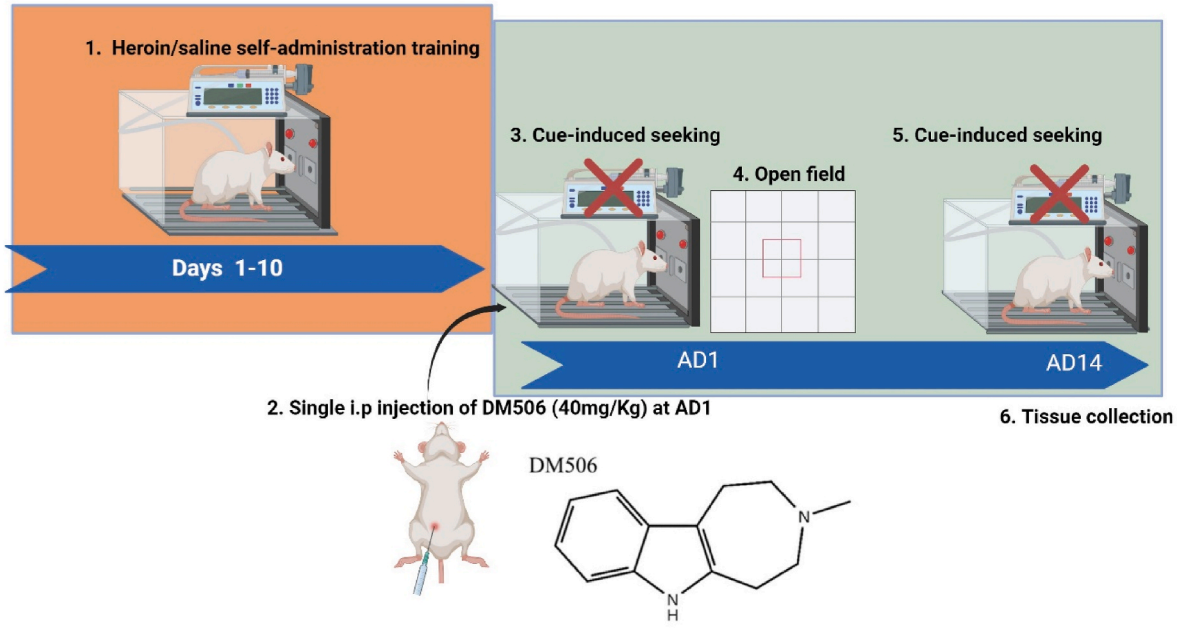

Fig. 1. Experimental timeline for heroin or saline self-administration, DM506 (3-methyl-1,2,3,4,5,6-hexahydroazepino[4,5-b]indole) injection, cue-induced seeking tests, and locomotor behavior. All events are numbered sequentially.

for slice electrophysiology (at the University of Kentucky). Animals in both locations were housed at 22–25 °C under a 12:12-h normal light–dark cycle with access to food and water ad libitum. All behavioral testing was conducted during the light phase of the light-dark cycle. All experiments were conducted in accordance with the Institutional Animal Care and Use Committees (IACUC) of Marshall University (behavioral experiments) and the University of Kentucky (slice electrophysiology).

2.2. Drugs

Heroin hydrochloride (Cayman Chemicals) dissolved in 0.9% sterile saline was used at a concentration of 0.02 mg/kg/infusion (inf) for self-administration studies. Heroin was delivered via syringe pumps, and injection volumes were adjusted daily according to body weight (Martin et al., 2019). As described previously, DM506 fumarate was synthesized using the Fischer Indole method, and DM506 fumarate obtained in the 2:1 ratio was dissolved in 1% DMSO. 1% DMSO was used as a vehicle (Tae et al., 2023). The dose of 40 mg/kg DM506 was determined based on previously published studies (Arias et al., 2024; Tae et al., 2023).

2.3. Jugular catheterization and patency testing

All rats were implanted with chronic indwelling jugular catheters as previously published (Mitra et al., 2021). Following anesthesia with ketamine and xylazine (60 and 5 mg/kg, respectively, intraperitoneally), an indwelling catheter was inserted into an isolated right jugular vein. The other end of the catheter was fitted to a vascular access harness (Instech, PA). Rats were allowed 5 days to recover from the surgical procedure, and catheter patency was preserved by flushing them daily with 0.2 mL of enrofloxacin (4 mg/mL) in heparinized saline (50 IU/mL in 0.9% sterile saline). One day before self-administration training, catheter patency was confirmed by loss of muscle tone and righting reflex following an intravenous infusion of ketamine hydrochloride (0.5 mg/kg in 0.05 mL). Only rats with patent catheters were used in behavioral studies.

2.4. Self-administration

Male and female rats were initially trained to self-administer heroin (or saline) and pseudorandomly divided into DM506 or vehicle treatment groups based on infusions in the last 3 days of self-administration (SA) training. A 3-h session each day of heroin SA for 10 days was employed at a dose of 0.02 mg/kg/infusion (pH = 7.4), which was delivered intravenously through a syringe pump contingent upon a response in the active nose poke operandum. Animals self-administering saline received sterile physiological saline (pH = 7.4) for the same infusion duration as heroin (and also received 3 h of access each day for 10 days). Following each infusion, the cue light located above the active nose poke was illuminated for 5-s and the house light was extinguished for 20-s. Animals began on a fixed ratio 1 (FR-1) on day 1 which was increased to FR2 (day 2) and FR3 (day 3) and maintained at FR3 for the remainder of the SA (Days 3–10). Animals that had at least 9 infusions of heroin on day 3 progressed forward for further training on days 4–10. We have previously established that this dose and exposure duration can achieve a stable escalation in heroin taking (Martin et al., 2019). Inactive nose poke responses were recorded throughout the sessions to control for non-specific responses. Responses to the inactive nose poke are provided in Table 1.

2.5. Cue-induced seeking

For cue-induced seeking behavior, rats were introduced to the same chambers where they had prior experience of taking the drug without any programmed consequences i.e., no delivery of the drug upon responses in the active nose pokes (Mitra et al., 2021; Werner et al., 2019, 2020). Animals were tested for cue-induced seeking behavior 2 h after the injections of 40 mg/kg DM506 or 1% DMSO vehicle on AD1. Cue-induced seeking was performed 2 h following injection based on a prior report of DM506's effect on reducing locomotor functions in the first hour following the injection (Arias et al., 2024). Animals were re-tested for cue seeking in AD14 to examine DM506's long-term effect on heroin-related behavior (Fig. 1). Total active responses during the 1-h test duration was used as a measure of cue-induced seeking.

Table 1
Inactive responses during self-administration.

Figure	Sex	Treatment	Inactive responses
Fig. 1a	Males	Saline + Vehicle	4.34 ± 0.38
		Saline + DM506	3.55 ± 0.22
		Heroin + Vehicle	3.1 ± 0.28
		Heroin + DM506	2.46 ± 0.32
Fig. 1b	Females	Saline + Vehicle	3.26 ± 0.24
		Saline + DM506	2.51 ± 0.28
		Heroin + Vehicle	3.1 ± 0.28
		Heroin + DM506	1.83 ± 0.29

2.6. Locomotor activity

Locomotor activity was quantified in transparent plastic cages using ANYMaze software that monitors the distance traveled. Locomotor activity was recorded during a 30-min test immediately following the cue-induced seeking behavior at AD1 (Fig. 1). We did not perform locomotor activity at AD14 since DM506 was administered only once at AD1.

2.7. Electrophysiological recording

2.7.1. Slicing

As previously published by our lab and others (Khatri et al., 2024; Sadanandan et al., 2020), rats were deeply anesthetized with isoflurane and brains were quickly removed following decapitation and placed into ice-cold cutting solution (cutting artificial cerebral spinal fluid, or ACSF) containing (in mM): 92 NMDG, 2.5 KCl, 1.25 NaH2PO4, 30 NaHCO3, 20 HEPES, 25 glucose, 2 thiourea, 5 Na-ascorbate, 3 Na-pyruvate, 0.5 CaCl2·2H2O, and 10 MgSO4·7H2O and saturated with carbogen (95% O2/5% CO2), pH:7.3–7.4, and osmolarity: 295 ± 5 mOsm. Brains were coronally sliced into 300 μm thickness sections containing the PrL by using a vibratome (Leica, VT1000S). Slices were immediately transferred to an oxygenated recovery chamber for ~30 min containing NMDG cutting solution and maintained at 32–34 °C. As described elsewhere (Ting et al., 2018), NaCl was gradually added to the recovery chamber. Following recovery, slices were transferred to a holding chamber maintained at room temperature containing holding solution (in mM: 92 NaCl, 2.5 KCl, 1.25 NaH2PO4, 30 NaHCO3, 20 HEPES, 25 glucose, 2 thiourea, 5 Na-ascorbate, 3Na-pyruvate, 2 CaCl2·2H2O, and 2 MgSO4·7H2O, 295 ± 5 mOsm and pH 7.3–7.40). Slices used for recording were maintained at room temperature for 4–6 h.

2.7.2. Recording

For recording, PrL coronal slices were gently transferred to a recording chamber that was continuously perfused with recording ACSF (in mM; 124 NaCl, 2.5 KCl, 1.25 NaH2PO4, 24 NaHCO3, 12.5 glucose, 5 HEPES, 2 CaCl2·2H2O, and 2 MgSO4·7H2O. pH to 7.3–7.4) at a flow rate of 1–2 ml/min. The PrL was visually located using a 10X air objective on an Olympus BX51WI fixed-stage upright microscope and layer IV-V neurons were identified by switching to a 60X water immersion objective using infrared DIC microscopy and ORCA-spark digital CMOS camera (Hamamatzu, NJ, USA). Whole-cell recordings were made from the soma of neurons using recording pipettes (4–7 mΩ) after establishing a giga-ohm seal (resistance range: 1–10 GΩ). Recording pipettes were made by pulling borosilicate glass pipettes (Sutter instruments, CA, USA) on Narishige pipette puller (Model PC-100,

Automate scientific, CA, USA) and filled with an intracellular solution containing (in mM): 135 CsCl, 10 HEPES, 4 MgCl2, 5 EGTA, 4 Na-ATP, 0.5 Na-GTP, 2 QX-314. Osmolarity was adjusted to 285 ± 5 mOsm, and pH was adjusted to 7.30 ± 0.01. Upon membrane rupture, cell membrane potential was held at −70 mV. Electrophysiological currents were recorded with a Multiclamp 700B amplifier (Molecular Devices, CA), filtered at 10 kHz, and digitized at 50 kHz. Data was collected using pCLAMP software (Molecular Devices) and analyzed offline using Igor-Pro (Wavemetrics, Lake Oswego, OR) with the Neuromatic toolkit (Rothman and Silver, 2018).

The tonic $GABA_A$ currents in PrL neurons were measured by the net change in holding current following the application of 100 μM Picrotoxin (Tocris, MN) or 10 μM DM-50 or both, as described in the results. The recording ACSF contained 10 μM NBQX and 50 μM DLAP5 to inhibit AMPA and NMDA currents respectively. Access resistances of all cells were monitored and cells deviating from 20 ± 10 MΩs were discarded.

2.8. Quantitative PCR

As previously published (Maulik et al., 2023; Mitra et al., 2021), quantitative polymerase chain reaction (qPCR) was performed on PrL tissues. Briefly, mRNA was isolated from 2 mm PrL tissue punches and purified using TRIzol (Ambion, Austin, TX) and the MicroElute total RNA kit (Omega Bio-tek Inc., Norcross, GA). 500 ng RNA was reverse transcribed to cDNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). For qPCR, reaction mixtures were prepared with iQ SYBR Green Supermix (Bio-Rad Laboratories) and run on an iQ5 system (Bio-Rad Laboratories). Reactions were run in duplicates, and *mRNA* expression was quantified using a relative threshold cycle method with *Gapdh* as a housekeeping gene. The primer sequences are provided in Table 2.

2.9. Statistical analysis

All statistical analyses were conducted using either JMP software from SAS or GraphPad Prism (GraphPad Software Inc., San Diego, CA). Performance during SA and cue-induced seeking data on AD1 and AD14 were analyzed using a liner mixed effects (LME) modeling (JMP software from SAS), with treatment groups (heroin + DM506, heroin + vehicle, saline + DM506, saline + vehicle) in both males and females.; Tukey's test was used for post hoc comparisons. For SA, the session was used as a random factor while treatment groups and sex were nested within the session. For cue-induced seeking, the subject was treated as a random factor and the treatment groups and sex were nested within the subject, while the abstinence days were treated as a continuous factor. The total number of nose-pokes per animal during the cue-induced seeking test recorded on AD1 and 14 was compared across treatment groups using LME. For both SA and cue-induced seeking, a model was chosen based on the lowest AiCC values. Two-way ANOVA was used to determine treatment, sex, and interaction effects in total distance traveled (at AD1) and *mRNA* expression (at AD14). The Grubbs test was applied to all data analysis for identifying outliers. No outliers were identified and thus no data points were excluded. Significance was set at a p-value of <0.05, and data were presented as the means ± SEMs. All graphs were plotted using GraphPad Prism 10.2.1.

3. Results

3.1. DM506 attenuates cue-induced seeking behavior at protracted abstinence from heroin SA

Rats self-administering heroin had significantly higher infusions than rats that earned saline infusions in both males and females (Table 3). Tukey's post hoc analysis showed that heroin SA was significantly higher than saline SA across all treatment groups. Heroin or saline SA in DM506 or vehicle treatment groups within males and females was not significantly different. However, we did observe that

Table 3
Statistical analysis for behaviors and mRNA expression.

Figure	Effect	p-value
Fig. 2a and b Self-administration	Treatment effect: $F_{(3,55)} = 117.07$	$p < 0.0001$
	Sex effect: $F_{(1,55)} = 59.37$	$p < 0.0001$
	Treatment x sex effect: $F_{(3,55)} = 2.96$	$p < 0.05$
Fig. 2c and d Cue-induced seeking behavior	Treatment effect: $F_{(3,55)} = 116.21$	$p < 0.0001$
	Sex effect: $F_{(1,55)} = 8.963$	$p < 0.01$
	Abstinence effect: $F_{(1,55)} = 87.63$	$p < 0.0001$
	Treatment x abstinence effect: $F_{(3,55)} = 26.92$	$p < 0.0001$
	Treatment x sex effect: $F_{(3,55)} = 5.13$	$p < 0.01$
	Abstinence x sex effect: $F_{(1,55)} = 5.75$	$p < 0.05$
	Treatment x abstinence x sex effect: $F_{(3,55)} = 2.79$	$p < 0.05$
Fig. 3 Locomotor behavior	Treatment effect: $F_{(3,55)} = 0.3926$	$p = 0.7588$
	Sex effect: $F_{(1,55)} = 1.218$	$p = 0.2745$
	F(1,55) = 87.63	$p < 0.0001$
	treatment x sex effect: $F_{(3,55)} = 1.091$	$p = 0.3607$
Fig. 5a Gene expression of $GABA_{A\alpha4}$	Treatment effect: $F_{(3,32)} = 0.4699$	p = 0.7054
	Sex effect: $F_{(1,32)} = 0.4435$	$p = 0.5104$
	Sex x treatment effect: $F_{(3,32)} = 0.2148$	$p = 0.8854$
Fig. 5b Gene expression of $GABA_{A\alpha5}$	Treatment effect: $F_{(3,32)} = 1.704$	p = 0.1859
	Sex effect: $F_{(1,32)} = 0.6897$	$p = 0.4124$
	Sex x treatment effect: $F_{(3,32)} = 1.039$	$p = 0.3885$
Fig. 5c Gene expression of $GABA_{A\alpha6}$	Treatment effect: $F_{(3,32)} = 1.122$	$p = 0.3546$
	Sex effect: $F_{(1,32)} = 1.239$	$p = 0.2740$
	Sex x treatment effect: $F_{(3,32)} = 1.216$	$p = 0.3198$
Fig. 5d Gene expression of $GABA_{A\delta}$	Treatment effect: $F_{(3,32)} = 0.3084$	$p = 0.8191$
	Sex effect: $F_{(1,32)} = 2.471$	$p = 0.1258$
	Sex x treatment effect: $F_{(3,32)} = 0.7639$	$p = 0.5227$

Table 2
Rat primers for amplifying $GABA_A$ subunit mRNA transcripts.

Primer	Forward (5′-3′)	Reverse (5′-3′)
$GABA_{A\alpha4}$	CCTTCTGGATCTGGCACAAGT	AATGCCCCAAATGTGACTGG
$GABA_{A\alpha5}$	CCATTTTTCCCAGCCAACAGA	TGTACCCGAGGATCTTTGCTTT
$GABA_{A\alpha6}$	AGCTGTATGCTTTGCGTTTGT	CTTTCGGCTTTCTGGGACTG
$GABA_{A\delta}$	ATGGCGCCAGAGCAATGAATGA	TTCTGAGATGTGGTCAATGCTGGC
Gapdh	AACGACCCCTTCATTGAC	TCCACGACATACTCAGCA

females self-administered more heroin than males. (Fig. 2a and b).

Cue-induced seeking in which rats respond to cues previously paired with heroin was used to evaluate cue-induced seeking behavior. We tested for cue-induced seeking at early (AD1) and prolonged abstinence (AD14) following heroin self-administration mainly to examine the long-term effects of a single administration of DM506, a pharmacological characteristic unique to psychoplastogens. Overall, an escalated cue-induced seeking was observed for heroin self-administered males and females at AD14 when compared to AD1 which aligns with the incubation effect in preclinical studies (Kuntz et al., 2008; Kuntz et al., 2008). Both male and female heroin self-administered rats that received a single dose of DM506 demonstrated reduced cue-induced seeking at AD1 and 14 in comparison to heroin self-administering rats receiving vehicle or saline self-administering rats receiving DM506 or vehicle [Fig. 2c and Table 3]). Post-hoc test revealed that the effect of DM506 on cue-induced seeking behavior extended to heroin SA males but not heroin SA females at AD14 (Fig. 2d and Table 3).

3.2. DM506 does not influence locomotor behavior

We also tested for locomotor behavior in an open field following the cue test at AD1 (Fig. 3). There was no significant treatment, sex, or treatment × sex interaction effect (Table 3) on the total distance traveled in the open field behavior of saline or heroin self-administered rats administered with vehicle or DM506.

3.3. DM506 inhibits tonic GABA currents within the PrL

In vivo microanalysis shows that a single dose of psychoplastogen, like psycobilin and ketamine, affects extracellular levels of DA, 5-HT, glutamate, and GABA in the rat frontal cortex (Wojtas et al., 2022). Additionally, there is evidence that psychedelics, which are parent compounds of psychoplastogens influence tonic current (Chiang and Aston-Jones, 1993). DM506 is shown to manifest its pharmacological effects via nicotinic and serotonin receptors, which are known to affect extracellular GABA in the PFC (Abi-Saab, 1999; Aracri et al., 2010).

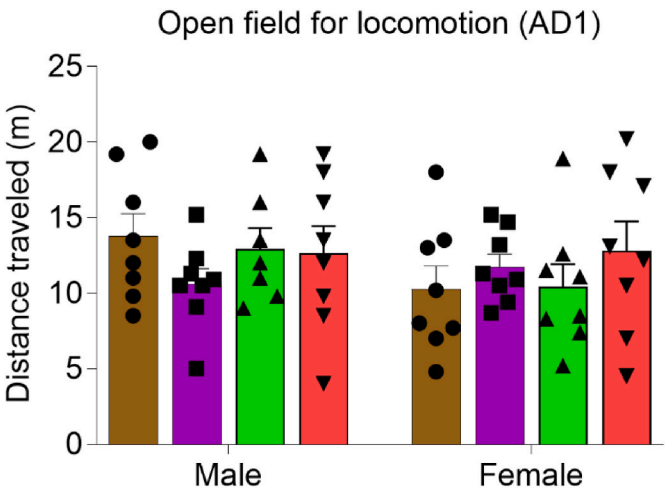

Fig. 3. Total distance traveled in a 30-min open field behavior test. n = 7–8 rats/group. All data are represented as means ± SEM.

Thus, we hypothesized that DM506 would modulate extracellular GABA and extracellular-GABA-mediated tonic currents. To test this, we used a whole-cell patch clamp to record tonic inhibition in Layer IV-V pyramidal neurons of the PrL region (Fig. 4a). First, we wanted to verify the presence of baseline tonic currents in the PrL. Tonic currents were calculated from the difference in holding current values before and after the application of $GABA_A$ receptor antagonist picrotoxin (PTX). Administering 100 μM of PTX in bath solution inhibited the IPSCs and decreased the holding current by 26.27 ± 7.62 pA, n = 6 (Fig. 4b, **e and f**). To test whether DM506 affects tonic currents in the PrL, we bath applied the slices with 10 μM DM506 for 5 min followed by PTX. We found that acute application of DM506 itself produced a small change in holding current (12.22 ± 6.13 pA, n = 9) that was not significant when compared to the control tonic currents. Subsequent PTX application induced a tonic current change of 18.41 ± 4.91 pA, whereas the total

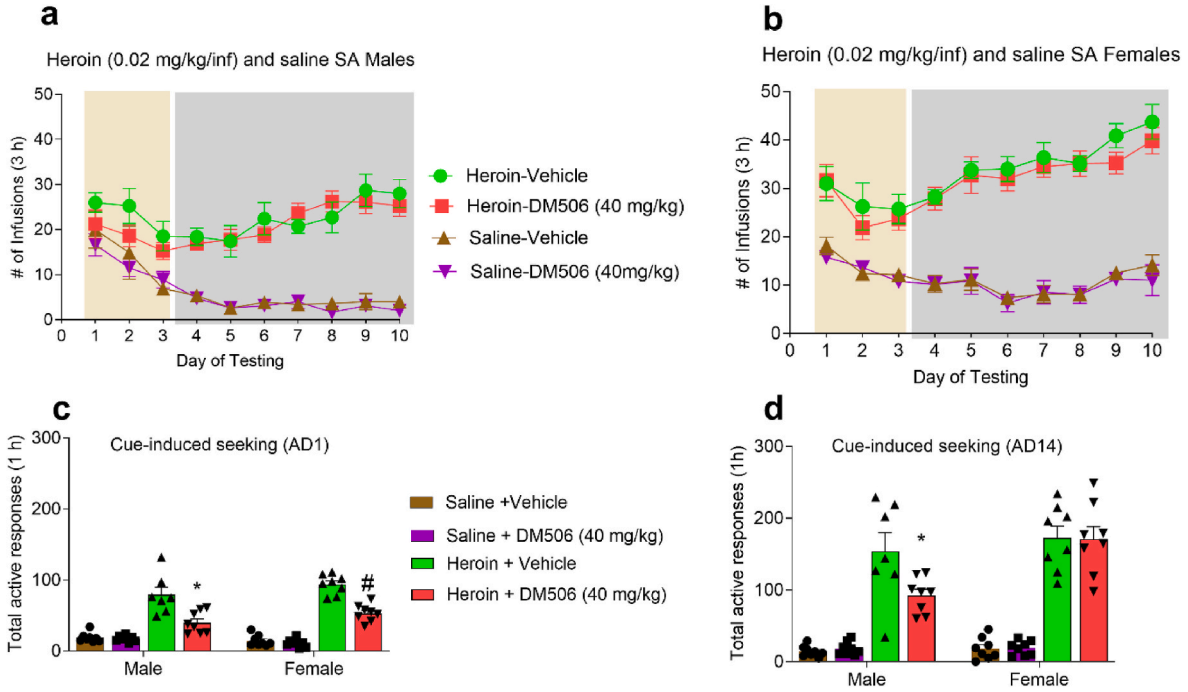

Fig. 2. Self-administration training for heroin and saline. Number of infusions for heroin or saline during 3 h of training in (a) males and (b) females. n = 7–8 rats/group. Total active responses in a 1-h cue-induced seeking test at (c) AD1 and (d) AD14. All data are represented as means ± SEM. *$p < 0.05$ between heroin males that received either vehicle or DM506 at AD1 and AD14. #$p < 0.05$ between heroin females that received either vehicle or DM506 at AD1.

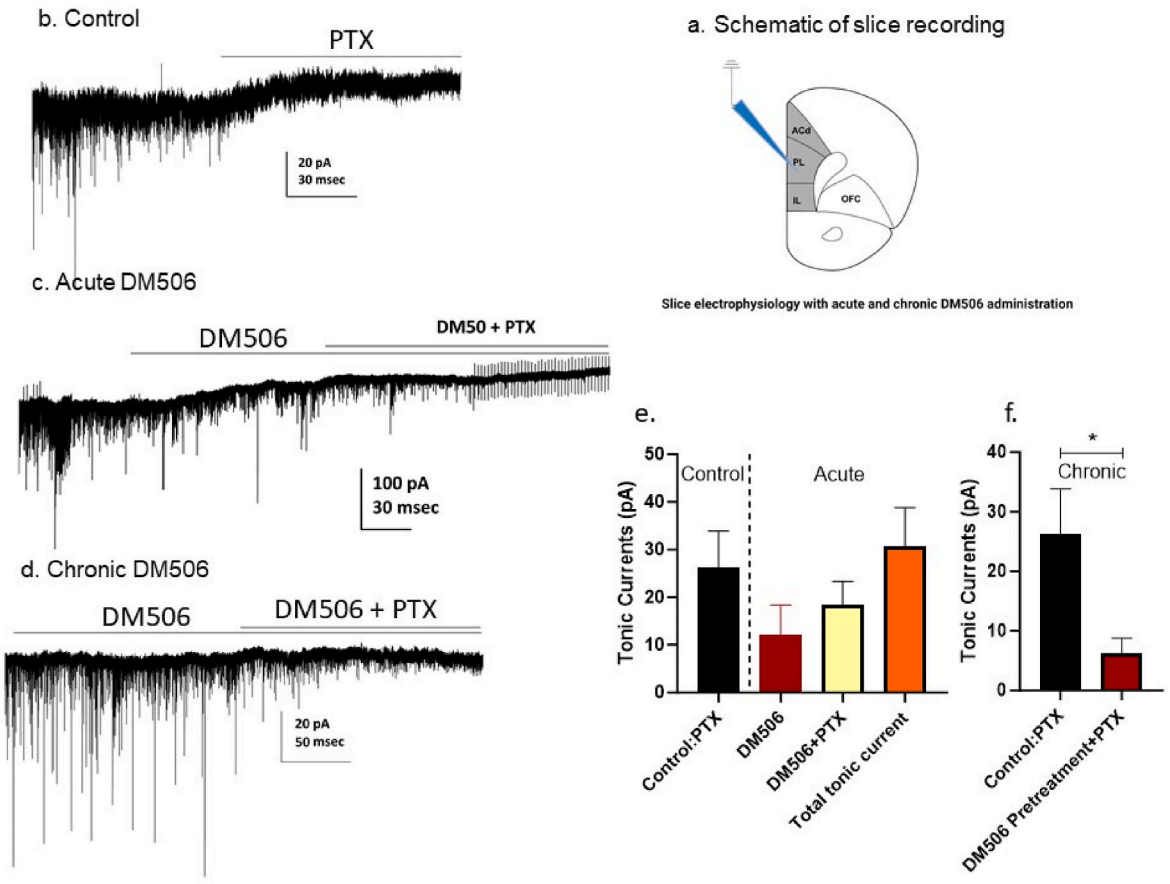

Fig. 4. DM506 inhibits tonic GABA current in the PrL. (a) Schematic of slice recording, showing patch electrode in the PrL region. (b) representative traces of baseline tonic current, deduced from the difference of holding current values before and after application of Picrotoxin (100 μM). (c) representative trace showing the change in tonic current change due to acute application of DM506 (10 μM) and PTX on PrL slices. (d) representative trace showing tonic current in slice preincubated in DM506 (10uM) and subsequent application of PTX. (e) Graphical representation of tonic currents during control and acute application of DM506. (f) Graphical representation of tonic currents after preincubation of slices in DM506, a chronic-like condition. n = 5–9 cells from 4 animals (2 per sex)/group. All data are represented as means ± SEM. *$p < 0.05$ between baseline tonic current with chronic DM506 incubation.

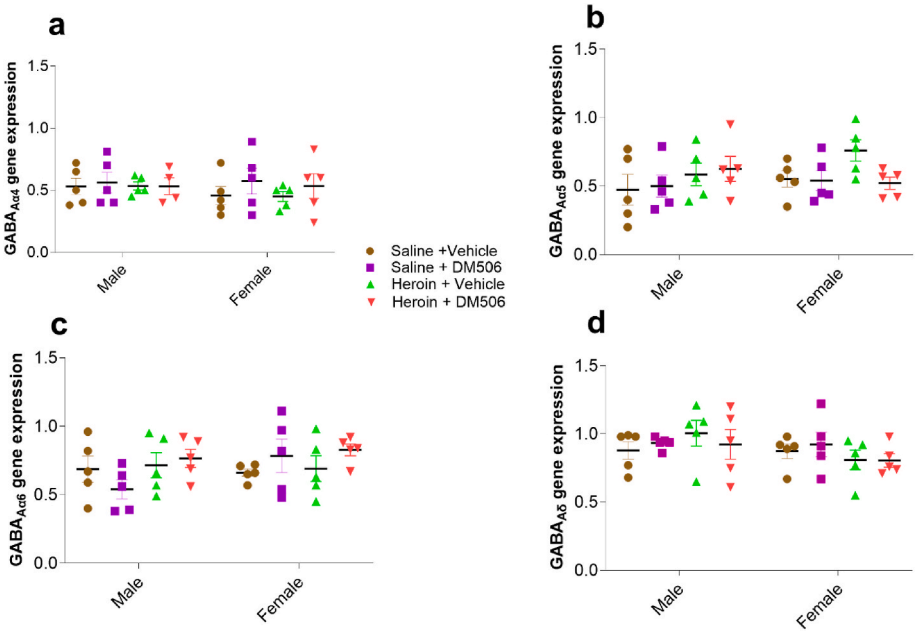

Fig. 5. DM506 does not alter $GABA_A$ receptor subunit gene expression. Gene expression changes of (a) α4 (b) α5, (c) α6, and (d) δ subunits of $GABA_A$ receptor at AD14 in the PrL of saline or heroin self-administered male and female rats administered with vehicle or DM506. n = 5/group. All data are represented as means ± SEM.

tonic current was still 30.63 ± 8.17 pA (Fig. 4c–e and f). This suggests that DM506 inhibits $GABA_A$ receptors and is either a weaker antagonist than PTX or may take a longer time to produce inhibition. Thus, to further study the effect of the chronic treatment of DM506 on tonic currents, we pretreated slices by incubating them in DM506 for 90–120 min. Post-pretreatment, application of PTX produced a minimal change in holding current (6.21 ± 2.55 pA, $n = 5$), showing that pretreatment of DM506 significantly reduced tonic GABA currents compared to baseline (*t-test*: $t_9 = 2.29$, $p < 0.05$ [Fig. 4d, e and f]). These data suggest that chronic application of DM506 decreases tonic currents by modulating extrasynaptic GABA levels or by indirectly inhibiting $GABA_A$ receptors.

3.4. DM506 does not alter gene expression of GABA subunits known to influence tonic current at AD14

We found no differences in treatment, sex, and sex x interaction effect on gene expression of $GABA_{A\alpha4}$, $GABA_{A\alpha5}$, $GABA_{A\alpha6}$, and $GABA_{A\delta}$ subunits in the PrL region of saline or heroin self-administered rats administered with vehicle or DM506 (Fig. 5 a-d and Table 3).

4. Discussion

Here we show that a novel psychoplastogen, DM506, attenuated cue-induced heroin seeking following protracted abstinence without impacting locomotor behavior. Our results indicated that a single injection of a dose of DM506 attenuated cue-induced seeking in both males and females at AD1. Further, there were no overall changes in locomotor activity due to DM506 administration demonstrating the specificity of this compound in reducing heroin-seeking behavior. These data are consistent with a prior report of unaltered locomotor activity with DM506 when tested 2 h after the systemic injection (Arias et al., 2024). DM506's effect on cue-induced heroin-seeking at AD1 and 14 parallels the acute and long-term effect of TBG administration on cue-induced reinstatement (Cameron et al., 2021). In another study, TBG reduced the progressive ratio for motivation in a contingent model of heroin and alcohol co-abuse (Heinsbroek et al., 2023). However, it is important to note that cue-induced seeking and cue-induced reinstatement are two separate behavioral modalities associated with heroin exposure and underlie discrete neurobiology (Feltenstein et al., 2021). Cue-induced seeking is performed under extinction conditions, while cue-induced reinstatement involves extinguishing the signal associated with drug cues followed by re-exposure of the cues. Similarly, the progressive ratio for motivation is reliant on the concept of behavioral economics (Giordano et al., 2001) and often engages distinct neurobiological changes (Feltenstein et al., 2021). Thus, a direct comparison cannot be drawn since we tested DM506's effect only on operant responses associated with drug-paired cues. Additionally, it is important to note that during acute conditions, TBG did not differentiate between heroin and sucrose seeking, perhaps, due to non-selective disruption of operant responding. Future studies will examine if this non-specific activity on drug and natural reward extends to other psychoplastogens such as DM506. Interestingly, the attenuating effect of DM506 on cue-induced seeking behavior was persistent only in males but not females at AD14, a time point when cue-induced seeking is known to exacerbate in rodents (Kuntz et al., 2008; Venniro et al., 2017). This raises the interesting possibility that sex could be an important determinant underlying the enduring effects of psychoplastogens. A prior clinical study has reported that race, ethnicity, and sex have a role in the prevalence of hallucinogen consumption, with Asian females exhibiting a higher prevalence than males and females of other races (Davis et al., 2022). The sex differences in cue-induced seeking found in the current study could be due to sex-specific metabolism as reported for other psychedelics (Highland et al., 2022; Saland and Kabbaj, 2018). As previously established, the pharmacological activity of DM506 is likely due to its activity on serotonin receptor 2A (5-$HT2_A$ receptor) (Arias et al., 2024) or nicotinic receptors (Aracri et al., 2010) or both. Since DM506 inhibits the α7 nicotinic acetylcholine receptors (Tae et al., 2023), we can discard the possibility that DM506-induced activation is involved as previously determined (Aracri et al., 2010). Conversely, sex differences in the $5HT_{2A}$ receptor expression profile could be involved. Sex differences have been observed in regulating $5HT_{2A}$ receptor expression resulting in a differential receptor availability profile for drug binding between males and females (Biver et al., 1996; Moses-Kolko et al., 2011; Soloff et al., 2010; Zhang et al., 1999). Though psychoplastogens affect the extracellular release of neurotransmitters, there is currently a paucity of mechanistic understanding of how these classes of drugs provide a differential therapeutic effect based on sex and warrants further examination.

The PFC is a critical neural hub that underlies the pathophysiology of substance use disorder due to its anatomic positioning to influence limbic reward circuits, attention, and top-down control over drug seeking (Goldstein and Volkow, 2011). Neuroimaging studies have demonstrated abnormal PFC function in patients afflicted with SUDs (Goldstein and Volkow, 2011). Opioids are known to impact the structure of cortical neurons, with reduced gray matter volume in the PFC being a hallmark of OUD (Goldstein and Volkow, 2011). These structural and functional abnormalities in the PFC are believed to worsen the disease by reducing executive control, exacerbating impulsivity, and impairing the extinction of drug-cue memories (Crews and Boettiger, 2009; Jentsch et al., 2014; Kalivas, 2008; Peters et al., 2009). Similarly, long-term neuroadaptations in the PFC regions are critical determinants of behavioral plasticity that underlie the pathophysiology of OUD (Peters and Olson, 2021). Additionally, studies have shown that the PFC, including the medial prefrontal cortex (mPFC), is one of the most important regions that undergo neuroplastic changes due to psychoplastogen exposure and might underlie its pharmacological effect (Browne and Lucki, 2013; De La Fuente Revenga et al., 2021; Li et al., 2010; Ly et al., 2018; Moda-Sava et al., 2019). PrL, which is encapsulated within the mPFC, is activated by heroin cues (Rubio et al., 2019). Therefore, pharmacological strategies aimed at restoring PrL structure/function are hypothesized to have the potential for reversing some of the opioid-induced neurobiological changes. Using PrL slices from drug-naive rats, we found that DM506 attenuated tonic GABAergic currents in the layer 4–5 pyramidal neurons when slices were pre-incubated in 10 μM DM506 as opposed to acute exposure. Our data corroborate prior evidence of the presence of tonic GABA currents in the PFC of both humans and rodents (Drasbek and Jensen, 2006; Scimemi et al., 2006; Sebe et al., 2010; Trujeque-Ramos et al., 2018; Yamada et al., 2004). This ambient/extracellular GABA is believed to be provided from several sources such as action potential-mediated (Bright et al., 2007) or non-synaptic release from astrocyte or reverse transport (Kozlov et al., 2006; Richerson and Wu, 2003). A recent study has shown that pyramidal neurons expressing $5HT2_A$ receptor regulate psilocybin-induced tonic firing in the mPFC,(Schmitz et al., 2022). DM506 activates the 5-HT_{2A} receptor (Arias et al., 2024), supporting our findings that the behavioral effects of psychoplastogens such as DM506 could be mediated by the modulation of tonic GABA currents. In turn, the decrease in tonic GABA might be mediated by 5-HT_{2A} receptors expressed in interneurons (Celada et al., 2013). However, our electrophysiological study was performed in heroin-naïve rats, limiting the interpretation of our slice recording results. Thus, future studies are needed to determine if DM506 impacts PrL GABAergic signaling following heroin self-administration. New evidence from our group indicates that ibogalogs inhibit γ-subunit containing synaptic $GABA_A$ receptors (data not shown; Arias et al., 2024 manuscript in final revision) with low potency in an in-vitro oocyte expression model. Though synaptic $GABA_A$ receptors do not contribute to tonic currents, it is plausible that DM506 inhibits extrasynaptic $GABA_A$ receptors. Intriguingly, we found that the *mRNA* levels of $GABA_A$ receptor subunits responsible for tonic currents remain unaltered in the DM506 or vehicle-treated saline and heroin SA animals at AD14. We could therefore rule out that the reduced tonic currents observed in the slice recordings might not be

linked to gene expression changes or inhibition of the GABAergic system and instead could be due to an increase in extracellular GABA by spill-over from GABAergic synapses or astrocytes. Experiments linking tonic GABA with DM506, and heroin-related behaviors are therefore needed to understand the extent of DM506 action. Another limitation of our study is the use of a single dose of DM506, and dose-dependent studies are needed to examine the boundary conditions underlying its pharmacological effect.

It is further noteworthy that though we have compared the cue-induced seeking data across early and prolonged abstinence, we do not intend to represent our data as an incubation phenomenon. We believe that the behavioral adaptations during these abstinence periods are discrete neurobehavioral events. Our data of enhanced cue-induced seeking among heroin self-administered males and females at AD1 and 14 though aligning with the prior preclinical literature (Kuntz et al., 2008), is likely not reflective of craving as clinically defined. Craving does not seem to exist reliably in opioid-experienced individuals (Bergeria et al., 2024). An increase in operant response in rats as a function of abstinence is most likely a behavioral outcome measure separate from the clinical concept of craving that needs to be better defined at the preclinical level of analysis.

5. Conclusion

In summary, our data indicate that DM506 has the potential to attenuate heroin-cue-related seeking behavior during abstinence from heroin SA. The current set of studies justifies future examination of the role of tonic GABA currents underlying psychoplastogen action on heroin-related behaviors. Further experiments are needed to determine mechanisms underlying sex-specific neurobiology impacting the efficacy of DM506 on heroin-induced behavioral outcomes.

Funding and credit

This study was funded by the PhRMA Foundation Faculty Starter grant in Drug Discovery and Marshall University Research Corporation Startup funds to SM. This work was also supported by the National Institutes of Health Grants DA055879, DA044479, DA046526, DA049130, and DA045881 to CDG. SM conceptualized the study with inputs from SNK, KL, and HRA. DM and MNR synthesized DM506. KL and CS performed behavioral experiments. MM, KC, AFC, and CM performed biochemical experiments. SNK performed electrophysiological recordings. SM and SNK wrote the manuscript with inputs from CDG and HRA.

CRediT authorship contribution statement

Kassandra Looschen: Investigation. **Shailesh Narayan Khatri:** Writing – review & editing, Methodology, Investigation. **Malabika Maulik:** Methodology, Investigation, Formal analysis, Conceptualization. **Colin Salisbury:** Investigation. **Alaina F. Carman:** Investigation. **Katilyn Corriveau:** Methodology. **Colton Smith:** Methodology. **Dina Manetti:** Methodology. **Maria Novella Romanelli:** Methodology. **Hugo R. Arias:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Cassandra D. Gipson:** Writing – review & editing, Supervision, Funding acquisition. **Swarup Mitra:** Writing – review & editing, Writing – original draft, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare no competing financial interests or personal relationships that could have influenced the current work.

Data availability

Data will be made available on request.

Acknowledgments

The authors would like to thank all animal care staff at the animal research facility (ARF) and Imaging Core User Facility at Marshall University School of Medicine.

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