

**TITLE: SEP-363856, A NOVEL PSYCHOTROPIC AGENT WITH A UNIQUE, NON-D₂
RECEPTOR MECHANISM OF ACTION**

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Running title: SEP-363856, a novel, non-D2 psychotropic agent

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Non-standard abbreviations:

MOA – Mechanism of action
LMA – locomotor activity
Tb – body temperature
NREM – non-rapid eye movement sleep
PPI – prepulse inhibition
VTA – ventral tegmental area
DRN – dorsal raphe nucleus
s.e.m. – standard error of the mean
s.d. – standard deviation

ABSTRACT

For the past 50 years, the clinical efficacy of antipsychotic medications has relied on blockade of dopamine D₂ receptors. Drug development of non-D₂ compounds, seeking to avoid the limiting side effects of dopamine receptor blockade, has failed to date to yield new medicines for patients. Here we report the discovery of SEP-363856 (SEP-856), a novel psychotropic agent with a unique mechanism of action. SEP-856 was discovered in a medicinal chemistry effort utilizing a high throughput, high content, mouse-behavior phenotyping platform, in combination with *in vitro* screening, aimed at developing non-D₂ (anti-target) compounds that could nevertheless retain efficacy across multiple animal models sensitive to D₂-based pharmacological mechanisms. SEP-856 demonstrated broad efficacy in putative rodent models relating to aspects of schizophrenia, including phencyclidine (PCP)-induced hyperactivity, prepulse inhibition and PCP-induced deficits in social interaction. In addition to its favorable pharmacokinetic properties, lack of D₂ receptor occupancy and the absence of catalepsy, SEP-856's broad profile was further highlighted by its robust suppression of rapid eye movement sleep in rats. Although the mechanism of action has not been fully elucidated, *in vitro* and *in vivo* pharmacology data as well as slice and *in vivo* electrophysiology recordings suggest that agonism at both trace amine-associated receptor 1 (TAAR1) and 5-HT_{1A} receptors are integral to its efficacy. Based on the preclinical data and its unique mechanism of action, SEP-856 is a promising new agent for the treatment of schizophrenia and represents a new pharmacological class expected to lack the side effects stemming from blockade of D₂ signaling.

SIGNIFICANCE STATEMENT (120 words max)

Since the discovery of chlorpromazine in the 1950's, the clinical efficacy of antipsychotic medications has relied on blockade of dopamine D₂ receptors, which is associated with substantial side effects and little to no efficacy in treating the negative and cognitive symptoms of schizophrenia. Here we describe the discovery and pharmacology of SEP-363856, a novel psychotropic agent that does not exerts its antipsychotic-like effects through direct interaction with D₂ receptors. Although the mechanism of action has not been fully elucidated, our data suggest that agonism at both TAAR1 and 5-HT_{1A} receptors are integral to its efficacy. Based on its unique profile in preclinical species, SEP-363856 represents a promising candidate for the treatment of schizophrenia and potentially other neuropsychiatric disorders.

INTRODUCTION

Schizophrenia is a chronic and disabling psychiatric disorder that affects approximately 1% of the global population. It is characterized by positive symptoms (eg. hallucinations, delusions and thought disorders), negative symptoms (eg. flat affect, anhedonia, alogia and avolition) and cognitive deficits (eg. impaired memory, attention and executive functioning). Despite advances in our understanding of the pathophysiology, schizophrenia remains one of the most challenging diseases to treat due to the diversity of clinical symptoms, the heterogeneity of clinical response, the side effects of current treatments and its association with high morbidity and mortality (Insel, 2010; Meyer-Lindenberg, 2010; Girgis *et al.*, 2018).

Antipsychotics have been the standard of care for schizophrenia since the discovery of chlorpromazine in the 1950s (Charpentier *et al.*, 1952; Laborit *et al.*, 1952; Lehmann and Ban, 1997). Since then, numerous “new” antipsychotics have been launched but they have essentially the same mechanism of action, mediating their efficacy against the positive symptoms through antagonism of dopamine D₂ and/or serotonin 5-HT_{2A} receptors. While improvements in drug safety have been made, a focus on the same molecular targets has not led to improved efficacy (Lieberman *et al.*, 2005; Girgis *et al.*, 2018). In fact, the negative and cognitive symptoms remain largely untreated by currently available antipsychotics. Furthermore, approximately 30% of patients have treatment-resistant schizophrenia (Samara *et al.*, 2016). The urgency for new treatments is therefore apparent.

More recently, drug development efforts have focused on molecular targets other than D₂ and 5-HT_{2A} receptors including GlyT1, D₁, D₄, D₃, NMDA, mGluR2/3, AMPA, 5-HT_{2C},

nicotinic $\alpha 7$, muscarinic M₁/M₄, H₃, NK-3, and sigma receptors (Miyamoto *et al.*, 2000; Karam *et al.*, 2010; Girgis *et al.*, 2018). However, despite promising efficacy in preclinical models for many of these targets, most novel, non-D₂/5HT_{2A} mechanisms have shown limited or no success in clinical trials (Girgis *et al.*, 2018). Thus, it is crucial to pursue alternative strategies for novel drug development for schizophrenia.

Traditional drug discovery efforts have been focused on designing compounds with high selectivity and potency for a target protein of interest. Unfortunately, in psychiatry there are few validated drug targets, in part due to the complexity of the disorders, rendering this approach largely unsuccessful. Phenotypic drug discovery (PDD) does not require any knowledge of a molecular target(s) associated with a disease and has been associated with the discovery of “first-in-class” medications (Swinney and Anthony, 2011; Moffat *et al.*, 2017). Examples include most anticonvulsants as well as antiviral drugs such as daclatasvir, which was discovered using a cell-based phenotypic screen (Belema and Meanwell, 2014). An *in vivo* PDD approach could be particularly valuable for the discovery of new therapeutics for psychiatric indications that have a complex underlying pathophysiology and where polypharmacology is common and likely necessary for clinical efficacy. However, the selection of an appropriate target combination and the design of a safe and efficacious polypharmacological molecule is extremely difficult. We therefore took a target-agnostic *in vivo* approach by utilizing a mouse behavioral platform (Roberds *et al.*, 2011; Alexandrov *et al.*, 2015; Shao *et al.*, 2016) together with anti-target *in vitro* screening to identify antipsychotic-like compounds that don't exert their effects through direct modulation of D₂ or 5-HT_{2A} receptors.

Here we report the discovery of SEP-363856 (SEP-856), a novel psychotropic agent with a unique, non-D₂/5-HT_{2A} mechanism of action. SEP-856 exhibits antipsychotic-like efficacy *in vivo* and demonstrates the potential for treating the positive and negative symptoms of schizophrenia. Although the mechanism of action has not been fully elucidated, *in vitro* and *in vivo* pharmacology data suggest that agonism at both 5-HT_{1A} receptors and TAAR1 are integral to its efficacy. The data presented here suggest that SEP-856 may have broad therapeutic efficacy in schizophrenia and potentially other psychiatric disorders.

MATERIALS AND METHODS

Animals

For behavioral screening, PCP-induced hyperactivity, catalepsy and prepulse inhibition (PPI) studies as well as patch-clamp recordings, adult male C57BL/6J mice were used. The forced swim test (FST) was performed in adult male BalbC/J mice. For the electroencephalogram (EEG) recordings, microdialysis, *in vivo* cellular recordings, autoradiography and PCP-induced deficits in social interaction, adult male Sprague Dawley rats were used. *In vivo* assessment of D₂ occupancy of SEP-856 was performed in non-human primate female baboons (*Papio Anubis*). *In vivo* pharmacokinetic studies were performed in male adult ICR mice, adult male Sprague Dawley rats and male Rhesus macaques (*Macaca mulatta*). Animals were maintained on a 12 /12 light/dark cycle. The room temperature was maintained between 20 and 23°C with a relative humidity maintained between 30% and 70%. Chow and water were provided ad libitum for the duration of the study unless otherwise stated. Animals were randomly assigned across treatment groups and studies conducted with experimenters blinded to the drug treatment. Further details (eg. Vendor, age and/or weight range) are provided in the Supplemental Information.

All animal studies were conducted in accordance with the institutional animal care protocols complying with Federal regulations and were approved by the respective Institutional Animal Care and Use Committees.

Test compounds

Haloperidol, Risperidone, Quetiapine, Clozapine, Sertraline, PCP, 8-OH-DPAT and WAY-100635 were purchased from Sigma Aldrich. SEP-856 ((S)-1-(4,7-dihydro-5H-thieno[2,3-c]pyran-7-yl)-N-methylmethanamine hydrochloride) and its enantiomer SEP-855 were synthesized by Sunovion Pharmaceuticals and all doses corrected for salt content. Further information regarding formulation for each study are provided in the Supplemental Information.

Behavior-based, mouse phenotypic screening

The central nervous system (CNS) properties of SEP-856 were evaluated using the SmartCube® system, a high-throughput, automated mouse behavioral platform (Roberds *et al.*, 2011; Alexandrov *et al.*, 2015; Shao *et al.*, 2016). Further experimental details are provided in the Supplemental Information.

Behavioral phenotyping

Details on PPI, PCP-induced hyperactivity, catalepsy, FST and PCP-induced deficits in social Interaction are provided in the Supplemental Information.

EEG recordings

EEG recordings were performed in 7 adult male Sprague Dawley rats using a cross-over design. Animals were implanted with chronic recording devices for continuous recordings of electroencephalograph (EEG), electromyograph (EMG), core body temperature (Tb), and locomotor activity (LMA) via telemetry (DQ ART 4.1 software; Data Sciences Inc., St Paul, MN). Following completion of the data collection, expert

scorers determined states of sleep and wakefulness in 10 second (s) epochs by examining the recordings visually using NeuroScore software (Data Sciences Inc., St Paul, MN). All doses of SEP-856, caffeine, and vehicle were administered by oral gavage. A minimum of 3 days elapsed between doses. In order to evaluate the effects of SEP-856 on sleep/wake parameters during the inactive period, dosing occurred during the middle of the rats' normal inactive period. The first 6 hours of the recording were scored and analyzed. For additional details, please refer to the Supplemental Information.

In vivo Microdialysis

Extracellular dopamine and serotonin levels were assessed in the prefrontal cortex and dorsal striatum using *in vivo* microdialysis in freely moving Sprague Dawley rats. For detailed methods, please refer to the Supplemental Information.

In vivo Pharmacokinetics studies

Details on *in vivo* pharmacokinetic measurements are provided in the Supplemental Information.

In vitro and *in vivo* 5-HT_{1A} and D₂ receptor occupancy studies

In vitro autoradiography was used to determine the effects of SEP-856 on [³H]-8-OH-DPAT binding to 5-HT_{1A} receptors in rat brain sections. *In vivo* occupancy of SEP-856 at D₂ receptors was measured with [³H]-raclopride in Sprague Dawley rats and with [¹⁸F]-Fallypride-PET in non-human primates (Papio Anubis). For details, please refer to Supplemental Information.

Patch-Clamp recordings in the DRN and VTA

In vitro whole-cell patch-clamp recording techniques were used in isolated slice preparations (male C57BL/6J mice, 4-16 weeks) of the dorsal raphe nucleus (DRN) and ventral tegmental area (VTA) to investigate the effects of SEP-856 on neuronal activity. The experiments examined the effects of SEP-856 (1-30 μ M) on the activity of DRN and VTA neurons that were characterized by their electrophysiological properties and their sensitivity to application of the 5-HT_{1A} receptor agonist 8-OH-DPAT (DPAT; 10 μ M). Subsequently, effects mediated via the trace amine-associated receptor 1 (TAAR1) and/or via the 5-HT_{1A} receptor were investigated using the selective antagonist EPPTB (0.05-1 μ M) and the selective antagonist WAY-100635 (WAY-635; 10 μ M) respectively. All compounds were dissolved in either DMSO or ddH₂O and diluted with aCSF to final concentration from a minimum 1000-fold higher stock concentration (maximum slice DMSO concentration 0.1%). Whole-cell patch-clamp recordings were performed at room temperature using the 'blind' version of the patch-clamp technique with either Axopatch 1D or Multiclamp 700B amplifiers. For detailed methods, please refer to the Supplemental Information.

In vivo extracellular single-unit recordings in the DRN

In vivo extracellular single-unit recordings were used to characterize the effects of SEP-856 on firing of serotonergic DRN neurons in anesthetized, male Sprague Dawley rats. Following surgery and insertion of the recording electrode, baseline firing activity of the neuron was recorded for at least 10 minutes prior to the compound administration. SEP-856 was tested at 1, 2, and 5 mg/kg by i.v. injection. After clear inhibitory effects were observed (3 – 5 minutes after compound administration), WAY-100635 (80 μ g/kg, i.v.)

was given to determine whether it could antagonize the inhibitory effect of SEP-856. Blood samples were taken 30 minutes following compound administration. For additional details, please refer to the Supplemental Information.

In Vitro Pharmacology

The *in vitro* pharmacology of SEP-856 at known receptors and enzymes was assessed in broad panel screens (Eurofins CEREP SA, Celle-Lévescault, France, Ricerca, Taipei Taiwan). For those targets at which SEP-856 (10 μ M) demonstrated greater than 50% inhibition, dose response curves were generated and inhibitory constant (K_i) values were determined. Equilibrium radioligand binding was carried out performed using the following incubation conditions: Incubation conditions and additional details are listed in the Supplemental Information.

The functional (both agonist and antagonist) effects were also determined. Assays used to study the functional effects were as follows: Intracellular cAMP levels were determined for 5-HT_{1A}, 5-HT₇, TAAR1 and D₂, using either the DiscoverX HitHunter cAMP XS+ assay or the Cisbio HTRF[®] cAMP assay. 5-HT_{1A} was also studied using GTP γ S binding. Impedance was used for 5-HT_{1B}, 5-HT_{1D}, and α_{2A} . Intracellular Ca²⁺ release was used for 5-HT_{2A} and 5-HT_{2C}. IP₁ accumulation was used for 5-HT_{2B}. D₂ was also studied using the DiscoverX PathHunter[®] β -arrestin recruitment assay.

Statistical Analyses

Statistical analyses were performed using the commercially available software GraphPad Prism v6.0, unless otherwise noted. Results are either presented as mean \pm s.e.m. or mean \pm s.d. (for PK analyses). Simple comparisons were evaluated with two-

tailed, Student's t-test. Multiple groups comparisons were assessed with One-way ANOVA, followed by appropriate post-hoc analyses. Time-dependent measures were assessed with repeated measures ANOVA followed appropriate post-hoc analyses. Statistical significance was defined as $p < 0.05$.

RESULTS

SEP-856 EXHIBITS ANTIPSYCHOTIC-LIKE ACTIVITY IN THE SMARTCUBE® SYSTEM

SEP-856 (Fig. 1A) was identified during a medicinal chemistry program designed to develop structurally and mechanistically novel antipsychotics using *in vivo* mouse phenotypic screening in combination with comprehensive *in vitro* and *in vivo* molecular profiling. Screening was conducted during a 45-minute automated test session, in which mice were exposed to multiple challenges, and different behavioral domains were captured and analyzed using proprietary computer vision software and machine learning algorithms (Roberds *et al.*, 2011; Alexandrov *et al.*, 2015; Shao *et al.*, 2016). The behavioral platform was established and validated with marketed CNS drugs, producing a library of drug-class signatures. Each class is represented by a different color, for example, purple and yellow indicate antipsychotic- and anxiolytic-like activity respectively (Fig. 1E), and the behavioral activity is shown as a scale of 0 to 100%. The data in Figure 1C demonstrated that SEP-856 was behaviorally active at three out of four doses tested (0.3, 1 and 10 mg/kg, i.p.). At 0.3 mg/kg, SEP-856 was classified as an anxiolytic (represented by the primarily yellow color of the column) but showed a dose-dependent increase in an antipsychotic classification (purple), such that the signatures at 1 and 10 mg/kg were predominantly antipsychotic. As a comparison, the signatures of marketed antipsychotic drugs are shown in Supplemental Figure 1. In addition, SEP-856 showed a modest antidepressant-like signal, illustrated by the green signal at 0.3, 1 and 10 mg/kg. Overall, the results indicate that SEP-856 is CNS active and exhibits a behavioral signature similar to known antipsychotic drugs. Interestingly,

its enantiomer, SEP-363855, showed little behavioral activity at 10 mg/kg (i.p.), a dose at which SEP-856 was fully efficacious (Fig. 1B and D).

IN VITRO PHARMACOLOGY

To investigate the molecular targets mediating the response to SEP-856, the compound was run against several panels of known molecular targets (ion channels, GPCRs and enzymes; Supplemental Tables 1-4). At 10 μ M, SEP-856 showed >50% inhibition of specific binding at α_{2A} , α_{2B} , D₂, 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}, and 5-HT₇ receptors. K_i values are shown in Table 1 ranging from 0.031 to 21 μ M. No significant activity of SEP-856 was observed at any of the enzymes studied (up to a concentration of 100 μ M).

Receptor panel screening and follow up functional testing showed that SEP-856 exhibited a range of activities at several receptors (Table 2). The most notable activity of SEP-856 was agonism at the human TAAR1 receptor (EC₅₀ of 0.14 \pm 0.062 μ M, E_{max} = 101.3 \pm 1.3%) and the 5-HT_{1A} receptor (EC₅₀ = 2.3 μ M with values ranging from 0.1- 3 μ M, E_{max} = 74.7 \pm 19.6%; Figure 2B). Interestingly, activity at the human TAAR1 receptor demonstrated stereoselectivity in that SEP-855 (an enantiomer of SEP-856), which was inactive in the behavioral screening platform at 10 mg/kg (i.p.), had an EC₅₀ of 1.7 μ M (Fig. 2A). In D₂ receptor functional assays, SEP-856 exhibited weak partial agonism with EC₅₀ values of 10.44 \pm 4 μ M (cAMP, E_{max} = 23.9 \pm 7.6 %; Fig. 2C) and 8 μ M (β -arrestin recruitment, E_{max} = 27.1%). At 100 μ M, 34 \pm 1.16 % inhibition was seen in the cAMP assay and no antagonism was seen at concentrations up to 100 μ M in the β -arrestin recruitment assay. Low potency partial agonist activities were also observed

at 5-HT_{1B} ($EC_{50} = 15.6 \pm 11.6 \mu\text{M}$, $E_{\text{max}} = 22.4 \pm 10.9 \%$), 5-HT_{1D} ($EC_{50} = 0.262 \pm 0.09 \mu\text{M}$ and $E_{\text{max}} = 57.1 \pm 5.5 \%$) and 5-HT₇ receptors ($EC_{50} = 6.9 \pm 1.32 \mu\text{M}$ and $E_{\text{max}} = 41.0 \pm 9.5 \%$). In a functional assay of 5-HT_{2B} activity, SEP-856 showed no agonism up to a concentration of 100 μM while norfenfluramine, the positive control, was a full agonist with an EC_{50} value of 0.140 μM . Little to no activity was detected at the 5-HT_{2A} receptor, with 29.3% agonism seen only at the highest tested concentration of 10 μM .

SEP-856 EXHIBITS HIGH BRAIN PENETRANCE AND GOOD SYSTEMIC BIOAVAILABILITY FOLLOWING ORAL ADMINISTRATION

Behavioral phenotypic screening demonstrated that SEP-856 is CNS active in mice following 0.3, 1 and 10 mg/kg intraperitoneal administration. Consequently, the pharmacokinetics of SEP-856 in plasma was characterized in ICR mice, Sprague Dawley rats and Rhesus macaques following oral and/or i.v. dosing. Biological samples were collected over 8 or 24 hours post-dose. Brain exposure was also assessed in mice and rats following p.o. administration. SEP-856 was rapidly absorbed with maximum plasma and brain concentrations reached within 0.25 to 0.5 hours in mice and rats and maximum plasma concentrations reached within 6 ± 2.83 hours in monkeys (Supplemental Table 5). SEP-856 penetrated mouse and rat brains after oral administration (10 mg/kg), with average brain-to-plasma AUC ratios of ~3 respectively (Supplemental Figure 2). In addition, SEP-856 plasma and brain levels were still detectable at 8 hours post dose with fairly consistent brain/plasma ratios over time. SEP-856's brain penetration and elimination pharmacokinetics as indicated by t_{max} and $t_{1/2}$ were similar to the plasma pharmacokinetics (Supplemental Table 5).

Oral bioavailability of SEP-856, determined by plasma AUC ratio after cross-over oral and intravenous administrations, was high in rat and monkey with 58-120% and ~71 % respectively. Total plasma clearance of SEP-856 was relatively high in rat (5 mg/kg, i.v.) and monkey (5 mg/kg, i.v.) with 1.54 and 0.797 L/h/kg, respectively, and elimination half-lives of 1.2 and 3.1 hours, respectively.

SEP-856 DEMONSTRATES ANTIPSYCHOTIC-LIKE EFFICACY IN RODENTS

In order to demonstrate the antipsychotic-like profile of SEP-856, we performed a series of additional pharmacological studies that assess endophenotypes of schizophrenia and antidepressant efficacy in rodents.

Acute treatment with phencyclidine (PCP), which induces robust hyperactivity in rodents and psychosis-like symptoms in humans, is considered a valuable assay in preclinical research and is widely used to screen novel compounds for antipsychotic efficacy (Ratajczak *et al.*, 2013; Steeds *et al.*, 2015; Moffat *et al.*, 2017). Single oral administration of SEP-856 (0.3, 1 and 3 mg/kg; 30 min pretreatment time) resulted in a dose-dependent inhibition of PCP-induced hyperactivity responses in C57Bl/6J mice (1-way ANOVA $F_{(5, 59)} = 18.96$, $p < 0.0001$; Tukey's post-hoc test, $p < 0.05$) with a 50% effective dose (ED_{50}) of approximately 0.3 mg/kg (Fig. 3A). The positive control, clozapine, also significantly reduced PCP-induced hyperactivity. Small but significant decreases in baseline activity were observed with SEP-856 at the highest dose of 3 mg/kg (1-way ANOVA $F_{(5, 59)} = 5.5$, $p < 0.001$; Tukey's post-hoc test, $p < 0.05$; Supplemental Figure 3A).

Another behavioral assay that is routinely used to identify novel antipsychotic agents is prepulse inhibition (PPI) of the acoustic startle response (Geyer *et al.*, 2001). PPI occurs when a startle-eliciting stimulus (i.e., the pulse) is preceded by a stimulus of lower intensity (i.e., the prepulse) and the amplitude of the startle response is reduced. Single oral administration of SEP-856 (0.3, 1, 3, 10, and 30 mg/kg; 30 minute pretreatment time) in C57BL/6J mice resulted in a dose-dependent increase in PPI compared to the respective vehicle treatment (Fig. 3B), with significant increases observed at 3, 10, and 30 mg/kg (2-way ANOVA: treatment effect $F_{(3, 102)} = 19.9$, $p < 0.0001$; db effect $F_{(2, 102)} = 30.97$, $p < 0.0001$; Dunnett's post hoc test, $p < 0.05$). Unlike the positive control Haloperidol, SEP-856 improved PPI at dose levels that had no confounding effects on baseline startle responses (Supplemental Figure 3B).

In contrast to positive symptoms, negative symptoms of schizophrenia, including anhedonia, blunted affect, and social withdrawal, are more difficult to model in animals (Jones *et al.*, 2011; Wilson *et al.*, 2015). However, subchronic treatment with PCP reliably produces social interaction deficits in rodents that may mimic certain aspects of negative symptoms such as social withdrawal (Steeds *et al.*, 2015). Sprague Dawley rats were subcutaneously injected with PCP (2 mg/kg) or vehicle twice daily for five consecutive days followed by behavioral assessment on day six. The PCP-induced deficit in social interaction was significantly attenuated by single oral administration of SEP-856 at 1, 3, and 10 mg/kg (1-way ANOVA $F_{(5, 56)} = 8.33$, $p < 0.0001$; Tukey's post-hoc test, $p < 0.05$). The positive control, clozapine (2.5 mg/kg, i.p.), also showed efficacy that was comparable to SEP-856 (Fig. 3C). This suggests that SEP-856 may

have potential benefits against some of the negative symptoms of schizophrenia such as social withdrawal.

The effects of SEP-856 (0.3, 1, 3, and 10 mg/kg) were additionally evaluated in the mouse FST, a behavioral assay that is acutely sensitive to all major classes of marketed antidepressant drugs (Porsolt *et al.*, 1977). Single oral administration of SEP-856 significantly reduced immobility time at 1, 3, and 10 mg/kg compared to vehicle (1-way ANOVA $F_{(5, 44)} = 13.24$, $p < 0.0001$; Tukey's post-hoc test, $p < 0.05$; Fig. 3D), suggesting that SEP-856 may also exhibit antidepressant-like activity. However, no clear dose-dependent effect was observed, and the magnitude of response was smaller than that produced by the positive control sertraline (20 mg/kg, i.p.).

One of the issues associated with antipsychotic drugs is the potential to develop extrapyramidal symptoms (EPS), which can be assessed in mice by measuring the induction of catalepsy using the bar test. Oral administration of SEP-856 (100 mg/kg) produced no effects in the bar test while haloperidol (1 mg/kg, i.p.) significantly increased the amount of time mice spent holding the bar at 30 and 90 min post dosing (2-way ANOVA: treatment effect $F_{(2, 54)} = 107.7$, $p < 0.0001$; time effect $F_{(1, 54)} = 9.4$, $p < 0.005$; Dunnett's post hoc test, $p < 0.05$). Importantly, these data indicate that SEP-856 was not associated with cataleptic effects at doses at least 30-fold higher than efficacious dose levels in mice (Fig. 3E).

The plasma and brain exposures to SEP-856 in the mouse PCP-induced hyperactivity and PPI tests as well as the rat subchronic PCP-induced social interaction test are shown in Supplemental Table 6. Taken together, our results indicate a clear

antipsychotic-like profile of SEP-856 in rodents and corroborate the initial findings obtained in the behavioral screening platform.

SEP-856 DECREASES RAPID-EYE-MOVEMENT (REM) SLEEP

5-HT_{1A} and TAAR1 activation have been shown to promote a generalized increase in wakefulness, increased latency to sleep, and suppression of REM sleep (F G Revel *et al.*, 2012; Black *et al.*, 2017; Schwartz *et al.*, 2017). Since SEP-856 exhibits full agonism at 5-HT_{1A} and TAAR1, we investigated whether it affects sleep architecture, as determined by telemetric recordings of the EEG, electromyogram, body temperature (T_b) and locomotor activity in Sprague-Dawley rats. SEP-856, and the positive control caffeine, were administered during the middle of the light (inactive) period followed by 6 hours of recordings. Oral SEP-856 administration (1, 3 and 10 mg/kg) produced a dose-dependent decrease in REM sleep, increase in latency to REM sleep and increase in cumulative wake (W) time (Fig. 4A, B and Supplemental Figure 4). SEP-856 had no effect on the cumulative non-REM (NREM) time and latency to NREM (Supplemental Figure 4). The differential effect on REM was further evident by a dose-dependent decrease in the REM:NR ratio (Fig. 4C). Caffeine promoted wakefulness as expected with additional characteristic increases in locomotion and T_b that SEP-856 did not produce (Supplemental Figure 4). Collectively, these results suggest that SEP-856 promotes vigilance when given during the light (inactive) phase.

SEP-856 INTERACTS WITH CENTRAL 5-HT_{1A} BUT NOT D₂ RECEPTORS

In addition to TAAR1, *in vitro* testing revealed that SEP-856 exhibits full and partial agonist activity at 5-HT_{1A} and D₂ receptors, respectively. Consequently, we conducted a

series of experiments to determine whether SEP-856 interacts with these two targets *in vivo*.

First, receptor autoradiography was used to assess SEP-856 occupancy at D₂ receptors in the rat brain using the radioligand [³H]-raclopride. SEP-856 (10 mg/kg, i.p.) or vehicle was administered to Sprague Dawley rats followed by administration of [³H]-raclopride (60 µCi/kg, i.v.) 30 minutes later. D₂ receptor occupancy was assessed at 60 minutes post SEP-856 (or vehicle) administration in coronal sections of the striatum (region of interest) and cerebellum (reference region). Despite high plasma (~1300 ng/ml), brain (~7900 ng/g), and CSF (~1800 ng/ml) exposures, SEP-856 resulted in 12.6 ± 6.4% receptor occupancy at D₂ (Supplemental Table 7), which was not statistically different from vehicle controls (two-tailed t-test, p = 0.27). For comparison, activity in the rat PCP-induced social interaction deficit assay (1 - 10 mg/kg) and mouse PCP-induced hyperactivity test (0.3 mg/kg – 3 mg/kg) was seen at much lower exposures (Supplemental Table 6). Thus, acute administration of SEP-856 did not produce significant occupancy at D₂ receptors in the rat brain at concentrations up to 200-fold greater than those that were behaviorally efficacious.

We additionally conducted *in vivo* Positron Emission Tomography (PET) imaging to determine whether SEP-856 also fails to occupy D₂ receptors in the non-human primate brain. Imaging was conducted in 2 anesthetized baboons using the radiotracer [¹⁸F]-Fallypride. The percent occupancy was evaluated using a blockade protocol comparing [¹⁸F]-Fallypride regional binding potential at baseline and following SEP-856 administration (~7.25 mg/kg, i.v. 30 minutes prior to [¹⁸F]-Fallypride injection). Venous blood samples were taken before and at various time points after SEP-856

administration. SEP-856 showed D₂ receptor occupancy levels of 9.1 ± 3.3 %, 6.2 ± 6.1 %, and 9.6 ± 8.8 % in the caudate, putamen, and globus pallidus, respectively (Supplemental Table 8 and Fig. 5). Similar to the observations in rats, SEP-856 did not produce significant occupancy at D₂ receptors despite achieving high plasma concentrations (i.e., 2850 ± 250 ng/ml at 60 minutes and 1765 ± 125 ng/ml at 180 minutes after SEP-856 administration). Importantly, this finding demonstrates that the antipsychotic-like behavioral profile of SEP-856 is independent of direct D₂ receptor modulation.

Next, we utilized *in vitro* autoradiography to evaluate the effects of SEP-856 on [³H]-8-OH-DPAT (a 5-HT_{1A} agonist radioligand) binding to 5-HT_{1A} receptors in the rat brain. Slide-mounted rat brain sections were incubated with 2 nM [³H]-8-OH-DPAT in the presence and absence of SEP-856 (0.1, 1 and 10 μM). Non-specific binding was defined by 10 μM 5-HT. SEP-856 produced a concentration-dependent displacement of [³H]-8-OH-DPAT binding in all regions evaluated (Supplemental Figure 5). Given the similar degree of [³H]-8-OH-DPAT in all brain areas, IC₅₀ values for SEP-856 displacement of [³H]-8-OH-DPAT binding were only determined for the septum and motor/somatosensory cortex (referred to as cortex). SEP-856 displaced [³H]-8-OH-DPAT in a concentration-dependent manner, with a mean IC₅₀ of 619 nM and 791 nM in the cortex and septum, respectively (Fig. 6). These results demonstrate that SEP-856 can bind to central 5-HT_{1A} receptor sites *in vitro*.

SEP-856 INHIBITS NEURONAL FIRING IN THE DORSAL RAPHE NEUCLEUS AND VENTRAL TEGMENTAL AREA *IN VITRO*

To investigate the effect of SEP-856 on neuronal activity and obtain further insights into its mechanism of action, we performed whole-cell patch-clamp recordings in isolated slice preparations of the DRN and the VTA of C57BL/6J mice. The DRN and VTA express a high abundance of 5-HT_{1A} and TAAR1 receptors, respectively (Lindemann *et al.*, 2008; Celada *et al.*, 2013; Christian and Berry, 2018). After the determination of repeatable responses, administration of SEP-856 was repeated in the presence of the selective TAAR1 antagonist EPPTB and/or the selective 5-HT_{1A} receptor antagonist WAY-100635.

Whole-cell current-clamp recordings were made from 29 neurons within the DRN with a mean resting membrane potential of -46.1 ± 1.0 mV and a mean input resistance of 849 ± 61 M Ω . Based on changes in membrane potential and firing rates, 16 of the DRN neurons were characterized as being sensitive to the 5-HT_{1A} agonist 8-OH-DPAT (DPAT), and 13 were classified as DPAT-insensitive (Supplemental Figure 6). Based on their initial threshold activity, 44% (7/16) of DPAT-sensitive neurons were characterized as spontaneously active (discharging action potentials) and 56% (9/16) were quiescent. SEP-856 (10 μ M) induced significant membrane hyperpolarization in 8 out of 9 quiescent neurons (45.5 ± 1.9 to -49.0 ± 2.2 mV, post-SEP-856 washout to 46.1 ± 1.9 mV; paired two-tailed t-test, $t_7 = 4.3$, $p = 0.004$; Fig. 7A). In contrast, one single neuron was depolarized by 2.1 mV. SEP-856 also significantly reduced the activity of 5 out of 7 spontaneously active DRN neurons (0.31 ± 0.10 Hz to 0.11 ± 0.04 , post-SEP-856 washout to 0.20 ± 0.06 Hz; $69 \pm 9.7\%$ reduction; paired two-tailed t-test, $t_4 = 3.2$, $p =$

0.03; Figure 7A), whilst the remaining two neurons were largely unaffected. Collectively, administration of 10 μ M SEP-856 induced an inhibitory response, determined by either a membrane hyperpolarization or a reduction in spontaneous firing rate, in 81% (13 out of 16) of DPAT-sensitive neurons. In contrast, SEP-856 only induced an inhibitory response in 15% (2 out of 13) of DPAT-insensitive neurons (Supplemental Figure 6).

Next, the effects of 10 μ M SEP-856 were examined in the presence of the 5-HT_{1A} antagonist WAY-100635 (1 μ M) in five DPAT-sensitive DRN neurons, two of which were spontaneously active and three of which were quiescent. In three of the five neurons, a membrane hyperpolarization of 1.9 ± 0.4 mV induced by SEP-856 was markedly reduced to 0.4 ± 0.1 mV when SEP-856 was reapplied in the presence of WAY-100635 (79% reduction, Fig. 7B and Supplemental Figure 6). Similarly, the inhibitory effect of SEP-856 was almost completely blocked in a further neuron in the presence of WAY-100635, with the spontaneous firing rate being reduced by 47% (0.21 to 0.11 Hz) during control SEP-856 administration compared to just a 2% reduction (0.83 to 0.82 Hz) when SEP-856 was applied in the presence of WAY-100635 (Fig. 7B). In the remaining neuron, a 2.4 mV hyperpolarization induced by SEP-856 was slightly increased to 2.6 mV in the presence of WAY-635. Thus, the inhibitory response induced by SEP-856 was reduced in the presence of the 5-HT_{1A} antagonist WAY-635, in the majority (4/5, 80%) of DRN neurons analyzed.

In addition, the effects of SEP-856 were examined in the presence of EPPTB in six DPAT-sensitive DRN neurons, three of which were spontaneously firing action potentials and three of which were quiescent. Overall, the inhibitory response induced

by SEP-856 was unaffected in the presence of the TAAR1 antagonist EPPTB, in the majority (5/6, 83%) of DRN neurons tested (Fig. 7C).

Following the assessment of SEP-856 effects on DRN neuronal firing, whole-cell patch-clamp recordings were also made from 23 neurons within the VTA (mean resting membrane potential of -40.7 ± 0.7 mV and a mean input resistance of 935 ± 104 M Ω) (Supplemental Figure 7). All of these neurons were spontaneously active once whole-cell configuration had been established with a mean firing rate of 1.97 ± 0.60 Hz, although activity did not persist in all cases. SEP-856 induced an inhibitory response in approximately half of the recorded VTA neurons (Fig. 7D). This response was characterized by a significant hyperpolarization of 2.8 ± 0.5 mV in 42% (8/19) of the neurons (-42.6 ± 1.3 mV to -45.3 ± 1.4 mV; paired two-tailed t-test, $t_7 = 5.8$, $p = 0.0007$) and a significant reduction in spontaneous firing rate in 55% (11/20) of the neurons (from 0.6 ± 0.18 to 0.18 ± 0.07 Hz; $71.4 \pm 7\%$ reduction; paired two-tailed t-test, $t_{10} = 3.3$, $p < 0.008$, Fig. 7D).

Interestingly, recordings from two neurons showed that the inhibitory response of SEP-856 (1 μ M) was reduced in the presence of 1 μ M EPPTB (Fig. 7F, Supplemental Figure 7), but persisted in the presence of the 5-HT_{1A} antagonist WAY-100635 (Fig. 7E). However, observations from additional neurons are necessary to further validate the ability of EPPTB to attenuate the inhibitory effect of SEP-856 in the VTA.

Overall, SEP-856 induced significant inhibitory responses in DPAT-sensitive neurons of the DRN and a subset of VTA neurons. In contrast to the inhibitory effects of SEP-856 in the DRN, which were primarily mediated via activation of 5-HT_{1A} receptors, the

response in the VTA appeared to be at least partially mediated via the activation of TAAR1.

SEP-856 INHIBITS DRN NEURONAL FIRING *IN VIVO*

The observation that SEP-856's inhibitory effects in the DRN were mediated by 5-HT_{1A} led us to additionally investigate DRN neuron firing *in vivo* by recording extracellular single-unit activities in anesthetized Sprague-Dawley rats. In accordance with previous work (Martin *et al.*, 1999), application of 8-OH-DPAT inhibited DRN firing, which was subsequently reversed by 0.08 mg/kg WAY-100635 (Supplemental Figure 8). SEP-856 (2 mg/kg, i.v.) significantly decreased DRN neuron discharges to 87% of the baseline rate (from 9.83 ± 1.4 to 1.4 ± 0.33 spikes/10 sec; two-tailed t-test, $t_2 = 5.9$, $p < 0.03$) during the initial 10 minutes post dosing (Fig. 8A,B). 30 minutes after SEP-856 administration the inhibitory effect was no longer detectable and firing-rates were restored to baseline levels. Plasma exposures determined 30 minutes post dosing were 422 ± 22.5 ng/ml. When SEP-856 was tested at a higher dose (5 mg/kg, i.v.), firing activity was completely suppressed and the inhibition was fully reversed by intravenous administration of 0.08 mg/kg WAY-100635 (Fig. 8C,D). Similarly, the inhibitory effect of 2 mg/kg SEP-856 was also reversed by WAY-100635 (Supplemental Figure 8). These results support the earlier *in vitro* finding in mice, and further suggest that part of SEP-856's mechanism of action is characterized by suppression of serotonergic neuronal firing via activation of 5-HT_{1A} autoreceptors.

SEP-856 DOES NOT ALTER DOPAMINE AND SEROTONIN RELEASE IN THE STRIATUM OR PREFRONTAL CORTEX

In order to determine whether the inhibitory action of SEP-856 in DRN neurons translates into changes in synaptic serotonin levels, we measured 5-HT release *in vivo* using microdialysis in freely-moving rats. Although SEP-856 demonstrated no occupancy at D₂ receptors *in vivo*, its' antipsychotic-like behavioral profile, TAAR1 activity and ability to inhibit VTA neurons suggest potential modulation of dopaminergic circuits independent of direct dopamine receptor regulation. We therefore also assessed dopamine release. Changes in monoamine levels were monitored over a 240-minute period following oral administration of vehicle, 3, 10, or 30 mg/kg SEP-856. Interestingly, no significant changes in extracellular levels of dopamine or 5-HT were observed in striatum or prefrontal cortex following SEP-856 administration (Supplemental Figure 9).

ANTIPSYCHOTIC-LIKE EFFECTS OF SEP-856 ARE PARTIALLY MEDIATED BY 5-HT_{1A} RECEPTORS

Although the microdialysis findings revealed no changes in SEP-856-mediated 5-HT release, the *in vitro* pharmacology results as well as the slice and *in vivo* electrophysiology data suggest that agonism at 5-HT_{1A} receptors is integral to SEP-856's mechanism of action in both mice and rats. Consequently, we evaluated whether the 5-HT_{1A} receptor antagonist WAY-100635 would attenuates the inhibitory behavioral effects of SEP-856 in the mouse PCP-induced hyperactivity test. C57BL/6J mice were injected with WAY-100635 (1 mg/kg, i.p.) or saline (i.p.) 10 minutes prior to SEP-856

dosing (3 mg/kg, oral) followed by PCP administration (5 mg/kg, i.p.) 30 minutes later. Consistent with our previous findings, single oral administration of SEP-856 significantly inhibited PCP-induced hyperactivity responses (Fig. 8E). Pretreatment with WAY-100635 partially attenuated the inhibitory effects of SEP-856 on the total distance traveled (1-way ANOVA $F_{(5, 51)} = 22.11$, $p < 0.0001$; Tukey's post-hoc test, $p < 0.05$). Taken together, the results indicate that the antipsychotic-like effects of SEP-856 in the PCP-induced hyperactivity test may be partially mediated through 5-HT_{1A} receptors.

DISCUSSION

Numerous advances have been made in understanding the potential role of receptors other than D₂ in contributing to drug efficacy and specific side effects in schizophrenia. Recent antipsychotic drug development has focused on non-D₂ targets including D₁, D₄, D₃, NMDA, 5-HT_{2A}, 5-HT_{2C}, M₁, M₄, H₃, NK₃, and sigma receptors (Miyamoto *et al.*, 2005; Karam *et al.*, 2010; Girgis *et al.*, 2018). However, thus far, no compound lacking D₂ receptor blockade has proven effective for any symptom dimension of schizophrenia. While several non-D₂ mechanisms, including mGlu_{2/3} receptor agonism and GlyT1 inhibition, have demonstrated efficacy in non-clinical and clinical proof-of-concept studies, positive results in Phase III clinical trials are still lacking (Marsman *et al.*, 2013; Kinon *et al.*, 2015; Girgis *et al.*, 2018). Here we utilized an *in vivo* mouse phenotypic screening platform in combination with comprehensive *in vitro* and *in vivo* profiling to identify compounds that exhibited behavioral similarity to known antipsychotics but do not act through D₂ or 5-HT_{2A} mechanisms. This led to the discovery of SEP-856, a potent compound that showed antipsychotic-like properties without inducing catalepsy, demonstrated potential antidepressant-like effects, suppressed REM sleep and modulated firing in a subset of VTA and DRN neurons.

In order to decipher the molecular MOA of SEP-856, its effects were initially studied in panel screens of large numbers of receptors, ion channels and enzymes. Subsequent studies identified activity at TAAR1, 5-HT_{1A}, 5-HT_{1D}, α_{2A} and D₂ receptors. Weak partial agonism of D₂ receptors (EC_{50} = 8-10 μ M, E_{max} 24-27%), and some very low potency antagonism (34% inhibition at 100 μ M), was seen in *in vitro* assays. It should be noted that this antagonism was assay dependent as, for instance, no antagonist response was

seen in the β -arrestin recruitment assay. Despite this *in vitro* activity, additional experiments indicated that the MOA of SEP-856 does not include *in vivo* blockade at D₂ given the lack of receptor occupancy in rat and monkey seen at doses/exposures that are efficacious in rodent behavioral assays. Furthermore, the demonstrated central occupancy of 5-HT_{1A} receptors, as well as the partial reversal of PCP-induced hyperactivity in the presence of WAY-100635, indicate that 5-HT_{1A} agonism contributes to the MOA of SEP-856. This was further supported by SEP-856's ability to induce significant inhibitory responses via 5-HT_{1A} receptors in putative serotonergic neurons in the DRN *in vitro* and *in vivo*. Notably, the inhibitory response induced in a subset of VTA neurons was mediated, at least in part, via activation of TAAR1, suggesting that SEP-856 modulates firing of putative serotonergic and dopaminergic neurons through distinct mechanisms.

TAAR1 is a GPCR activated by trace amines and expressed in multiple regions of the mammalian brain. The highest expression is reported in the DRN and VTA, whereas lower levels are observed in the limbic system and several other brain areas (Borowsky *et al.*, 2001; Burchett and Hicks, 2006; Lindemann *et al.*, 2008; Rutigliano *et al.*, 2018). Recent studies have extensively characterized TAAR1 functions, elucidating its important role in modulating dopaminergic circuitry and its potential implications in neuropsychiatric disorders (Borowsky *et al.*, 2001; Leo *et al.*, 2014; Gainetdinov *et al.*, 2018; Schwartz *et al.*, 2018). Revel *et al.* reported the first selective TAAR1 agonist (RO5166017) and demonstrated its inhibitory effects on the firing of dopaminergic and serotonergic neurons (Revel *et al.*, 2011). In addition, the antipsychotic-like profile of full and partial TAAR1-selective agonists was shown by their ability to inhibit cocaine and

PCP-induced hyperlocomotion in rodents despite their lack of affinity for D₂ receptors (Revel *et al.*, 2011; F G Revel *et al.*, 2012; Florent G. Revel, Moreau, *et al.*, 2012). Similarly, SEP-856 was able to block PCP-induced hyperactivity without significantly occupying D₂ receptors. Unfortunately, the lack of suitable TAAR1 antagonists prevented direct assessment of TAAR1's contribution to the antipsychotic-like activity of SEP-856 *in vivo*.

TAAR1's ability to regulate presynaptic dopaminergic neurotransmission makes it an interesting target for a number of psychiatric disorders (Lindemann *et al.*, 2008; Revel *et al.*, 2011; Leo *et al.*, 2014). Along these lines, TAAR1 agonists have demonstrated efficacy in genetic mouse models of hyperdopaminergia, including DAT knockout (KO) mice and rats (Revel *et al.*, 2011; Florent G. Revel, Meyer, *et al.*, 2012; Florent G. Revel, Moreau, *et al.*, 2012). The observed effects on dopaminergic signaling presumably occur via functional physical interaction of TAAR1 with D₂ receptors and potentially also with the dopamine transporter (Espinoza *et al.*, 2011; Leo *et al.*, 2014; Harmeier *et al.*, 2015; Leo and Espinoza, 2016). Cell culture studies have shown that TAAR1 is normally located intracellularly but can translocate to the plasma membrane when co-expressed with D₂ receptors (Espinoza *et al.*, 2011; Harmeier *et al.*, 2015). The ability of TAAR1 to modulate dopaminergic tone was also demonstrated in TAAR1 KO mice. Under baseline conditions, striatal dopamine release was not altered in KO animals compared to wild-type controls but was augmented following an amphetamine challenge (Wolinsky *et al.*, 2007; Lindemann *et al.*, 2008). Follow up work by Leo and colleagues, using microdialysis and fast-scan cyclic voltammetry, revealed increased levels of dopamine specifically in the nucleus accumbens but not in the dorsal striatum

of TAAR1 KO mice (Lindemann *et al.*, 2008; Leo *et al.*, 2014). In accordance, TAAR1 agonists have been reported to suppress VTA neuronal firing (Revel *et al.*, 2011) and inhibit electrically-evoked dopamine release (Leo *et al.*, 2014). SEP-856 exerted inhibitory effects in VTA neurons, which were likely mediated via activation of TAAR1. Interestingly, the inhibitory response was only observed in half of the recorded cells, pointing to a selective suppression of a subset of VTA neurons by SEP-856. The lack of D₂ occupancy, in addition to the inhibitory effects on VTA neuronal firing, potentially suggest modulation of presynaptic dopamine dysfunction by SEP-856. Abnormalities in dopamine synthesis capacity, baseline synaptic dopamine levels, and dopamine release have been reported in schizophrenia patients, and are not targeted by current antipsychotic treatments (Howes *et al.*, 2012; Jauhar *et al.*, 2017; Kim *et al.*, 2017; McCutcheon *et al.*, 2018). Although SEP-856 demonstrated clear inhibitory effects on DRN and VTA neurons, it did not alter dopamine or 5-HT release in the striatum or prefrontal cortex. This could be due to a number of reasons including the difficulty in detecting potential decreases in neurotransmitter release under baseline conditions using conventional microdialysis. Differential effects of SEP-856 on dopamine and/or serotonin release might primarily be detectable under stimulated conditions (e.g. following electrical, pharmacological or behavioral challenges) or in a disease context (e.g. hyperdopaminergic state). On the other hand, changes in dopaminergic and serotonergic cell firing do not necessarily translate into changes in dopamine/5-HT release (Berke, 2018). For example, dopamine release can be locally controlled by the presynaptic terminals themselves and thus show spatiotemporal patterns independent of cell body spiking (Floresco *et al.*, 1998; Jones *et al.*, 2010; Berke, 2018). In addition,

SEP-856 might affect tonic versus phasic dopaminergic firing differentially, which could ultimately result in marked behavioral effects without necessarily altering overall dopamine levels. Additional experiments, applying both microdialysis and fast-scan cyclic voltammetry will help to further elucidate the effect of SEP-856 on neurotransmitter release and firing kinetics under baseline and stimulated conditions.

In addition to TAAR1-mediated activity, 5-HT_{1A} agonism was identified as an integral part of the MOA of SEP-856. The 5-HT_{1A} receptor is highly expressed in the DRN, cortex and limbic forebrain areas (e.g. hippocampus and amygdala), with lower densities detected in the basal ganglia, thalamus, substantia nigra and VTA (Kia *et al.*, 1996; Hiroshi Ito, 1999; De Almeida and Mengod, 2008). In the DRN, 5-HT_{1A} receptors are primarily somatodendritic autoreceptors which function to inhibit neuronal firing. In contrast, they are present as postsynaptic receptors in the hippocampus and amygdala (Celada *et al.*, 2013). Thus, it will be interesting to test whether SEP-856 also acts in an inhibitory fashion in forebrain regions such as the hippocampus or prefrontal cortex.

The serotonergic circuitry has repeatedly been implicated in the pathophysiology of schizophrenia, which is in part due to the antipsychotic properties of 5-HT_{2A} antagonists (although primarily in combination with D₂ blockers). In addition, 5-HT_{2C} antagonists and compounds that target both receptors 5-HT_{2A}/5-HT_{2C} (e.g. ritanserin, vabicaserin, mianserin, SR46349B, etc.) have demonstrated some efficacy in schizophrenia (Girgis *et al.*, 2018). Although there is multiple evidence for the therapeutic efficacy of 5-HT_{1A} agonists in depression and anxiety, less is known about the potential contribution of these receptors to schizophrenia. Postmortem (Burnet *et al.*, 1996, 1997; Simpson *et al.*, 1996; Sumiyoshi *et al.*, 1996) and neuroimaging studies (Kasper *et al.*, 2002;

Tauscher *et al.*, 2002) have revealed alterations in 5-HT_{1A}-receptor density in the cortex and amygdala of schizophrenic patients (Yasuno *et al.*, 2004). Evidence in rodent models indicates that the activation of 5-HT_{1A} receptors prevents EPS induced by D₂ receptor blockade, modulates dopaminergic neurotransmission in the frontal cortex, positively influences mood, and attenuates NMDA receptor antagonist-induced cognitive and social interaction deficits (Newman-Tancredi, 2010; Celada *et al.*, 2013). In addition, a number of compounds that combine partial agonism at 5-HT_{1A} receptors with antagonism (or partial agonism) at D₂ receptors (e.g. aripiprazole, perospirone, lurasidone, cariprazine, PF-217830, F-97013-GD, F-15063 and bifeprunox) appear to provide therapeutic benefits against a broader range of schizophrenia symptoms (Newman-Tancredi, 2010; Celada *et al.*, 2013).

Interestingly, earlier work demonstrated that modulation of TAAR1 activity via the selective agonist RO5166017 increases the potency of 5-HT_{1A} partial agonists and alters the desensitization rate at the 5-HT_{1A} autoreceptors in the DRN (Revel *et al.*, 2011). This can occur by direct interaction of TAAR1 and 5-HT_{1A}, or by favoring interactions with the GPCR desensitization machinery. Consequently, Revel and colleagues proposed that co-treatment with a TAAR1 agonist might improve therapeutic efficacy of classical antidepressants, providing further support for compounds with dual 5-HT_{1A}/TAAR1 activity in the treatment of psychosis and mood. Notably, TAAR1 agonists and many antidepressants (especially those with 5-HT_{1A} activity) exert REM sleep suppression, which is not observed for most D₂-based antipsychotics (Tribl *et al.*, 2013; Wichniak *et al.*, 2017; Goonawardena *et al.*, 2019). Thus, the robust REM suppression observed with SEP-856 could result from synergistic action on TAAR1 and

5-HT_{1A} receptors. However, agonism at TAAR1 and 5-HT_{1A} receptors might only partially contribute to SEP-856's MOA, with other (possibly not yet identified) targets likely playing key roles as well.

Although this work made use of multiple pharmacological rodent models and assays, it is important to mention that no animal model is, or ever will be, truly reflective of the underlying disease etiology of schizophrenia. Nevertheless, certain models serve as important investigational tools and are useful in the development of novel treatments, especially when the goals for a specific model and/or approach are clearly defined (Nestler and Hyman, 2010). Our goal was to identify a novel MOA for the treatment of schizophrenia by focusing on a desired clinical outcome – the combination of antipsychotic efficacy and lack of D2-mediated side effects (e.g. EPS and endocrine effects). Ultimately, proof-of-concept studies in humans are required to determine whether preclinical findings will translate into therapeutic efficacy. SEP-856 is currently being evaluated for the treatment of schizophrenia in randomized controlled clinical trials.

In summary, the approach utilized here represents an alternative to target-driven drug discovery as it relied on *in vivo* phenotypic and *in vitro* (anti-target) screening followed by subsequent verification in putative animal models of schizophrenia. Accordingly, the MOA of SEP-856 has not been fully elucidated. However, the selective agonism of TAAR1 and 5-HT_{1A}, in addition to the lack of D₂/5-HT_{2A} mediated efficacy, has the potential to translate into a significantly improved safety profile compared to available therapies, while still maintaining antipsychotic efficacy across a broad array of symptoms. Based on its unique MOA and preclinical profile in animals, SEP-856

represents a promising candidate for the treatment of schizophrenia and potentially other neuropsychiatric disorders.

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FOOTNOTES

At the time these studies were conducted, all authors were either employees of Sunovion Pharmaceuticals or Psychogenics Inc. Some authors are inventors on patents related to the subject matter.

FIGURE LEGENDS

Figure 1. SEP-856 exhibits a predominantly antipsychotic-like signature in SmartCube®. The chemical structures of SEP-856 (A) and its enantiomer SEP-855 (B). The behavioral signature of SEP-856 (C) includes anxiolytic (yellow) and antipsychotic (purple) components. In contrast, its enantiomer SEP-855 is largely behaviorally inactive in mice, represented by the vehicle-like white bar (D). The behavioral platform was established and validated with marketed CNS drugs, producing a library of drug-class signatures. (E) Each of the 15 classes is represented by a different color, as indicated (*antipsychotic (purple) and high-dose antipsychotic (dark purple); **antidepressant (green) and *high-dose antidepressant (dark green)). N = 8-10 mice/group.

Figure 2. Functional effects of SEP-856 at TAAR1, 5-HT_{1A} and D₂ receptors. The functional effects of SEP-856 were determined using HEK-293 cells expressing TAAR1 (A), 5-HT_{1A} (B) or D₂ (C) receptors. cAMP accumulation was determined for TAAR1 using the DiscoverX HitHunter cAMP Assay. The inactive enantiomer of SEP-856 (SEP-855) was also tested along with the trace amines PEA and p-tyramine. Inhibition of forskolin-stimulated cAMP levels was used for the 5-HT_{1A} (n = 4) and D₂ receptors; cAMP levels were determined with the DiscoverX HitHunter cAMP XS+ assay or the Cisbio HTRF®, cAMP assay. The D₂ receptor was also studied using the PathHunter β-arrestin recruitment assay. Representative traces are shown for A and C.

Figure 3. SEP-856 demonstrates antipsychotic- and antidepressant-like activity without inducing catalepsy. (A) Oral SEP-856 administration dose-dependently reduced PCP-induced hyperactivity in C57BL/6J mice (1-way ANOVA + Tukey's post hoc test, * p < 0.05 vs. Veh/PCP, #p < 0.05 vs Veh/Veh). Clozapine and PCP were dosed at 1 mg/kg and 5 mg/kg i.p., respectively. (B) PPI measured in C57BL/6J mice was significantly increased by Haloperidol (i.p.) and SEP-856 at 3, 10 and 30 mg/kg (p.o.) compared to their respective vehicle controls (10 % DMSO for Haloperidol and 20% cyclodextrin for SEP-856; 1-way ANOVA + Tukey's post hoc test, *# p < 0.05). (C) Social interaction deficits induced by subchronic PCP treatment (2.5 mg/kg, s.c., twice daily for 5 days) in Sprague-Dawley rats were significantly attenuated by acute

Clozapine (2.5 mg/kg, i.p.) and SEP-856 (p.o) treatment at all doses tested (1-way ANOVA + Tukey's post hoc test, * $p < 0.05$ vs. PCP/Veh, # $p < 0.05$ vs Veh/Veh). (D) Acute Sertraline (20 mg/kg, i.p.) and 1, 3 and 10 mg/kg SEP-856 (p.o.) dosing reduced immobility time in the FST in Balb/CJ mice compared to vehicle controls (1-way ANOVA + Tukey's post hoc test, * $p < 0.05$ vs. Veh). (E) In contrast to Haloperidol (i.p.), acute SEP-856 (p.o.) treatment did not induce catalepsy in the mouse (C57BL/6J) bar test as seen by the lack of increase in time spent holding the bar compared to vehicle (2-way ANOVA + Tukey's post hoc, * $p < 0.05$ vs. Veh). Abbreviations: CLZ, clozapine; SRT, sertraline; PCP, phencyclidine; PPI, prepulse-inhibition; FST, forced-swim test. N = 8-12/group. Data are shown as mean \pm s.e.m.

Figure 4. SEP-856 decreases REM sleep in rats. (A) Acute, oral administration of SEP-856 led to a dose-dependent reduction in cumulative REM sleep (A), increase in latency to REM (B) and a decrease in REM/NREM ratio (C) in Sprague-Dawley rats over the 6-hour recording period (1-way repeated-measures ANOVA + two-tailed t-test, * $p < 0.05$ vs. Veh). Dosing occurred in the middle of the inactive phase, at the beginning of Zeitgeber time 7. The positive control Caffeine (10 mg/kg, p.o. CAF) produced similar effects on REM time and latency without altering the REM/NREM ratio. Additional parameters are presented in Supplemental Figure 4. N = 7. Data are shown as mean \pm s.e.m.

Figure 5. SEP-856 does not exhibit significant occupancy at D₂ receptors in non-human primates. Representative MR (for anatomic reference) and mean summed PET images, before and after administration of SEP-856 are shown from top to bottom for one animal. Axial, sagittal, and coronal slices are shown from left to right. Regions of interest, including the caudate, putamen and globus pallidus, were manually outlined. Average [¹⁸F]Fallypride PET images over 180 minutes of acquisition are presented at baseline and following bolus i.v. administration of SEP-856 (7.25 mg/kg). The occupancy was estimated using the BP_{ND} -derived Simplified Reference Tissue Model (SRTM). SUV_r, relative standardized uptake value.

Figure 6. SEP-856 binds to 5-HT_{1A} receptors in rat brain slices. (A) Autoradiographs of coronal brain sections depicting binding of the 5-HT_{1A} agonist [³H]-8-OH-DPAT. The highest receptor binding was observed in the septum and throughout the cortex (illustrated by increasing intensity in yellow to red colors). (B) SEP-856 displaced [³H]-8-OH-DPAT in a concentration-dependent manner, with an IC₅₀ of 619 nM and 791 nM in the cortex and septum, respectively (n = 4 brains, 2 sections/brain). Sections were incubated with 2 nM [³H]-8-OH-DPAT and 10 μM serotonin for NSB (non-specific binding), 0.1% DMSO for total binding or SEP-856 (0.1 - 32 μM). Data are shown as mean ± s.e.m.

Figure 7. SEP-856 inhibits firing of DRN and VTA neurons *in vitro*. Representative whole-cell patch-clamp recordings from DRN and VTA brain slices of C57BL/6J mice are shown on the left, and quantification histograms on the right. Compound-induced effects were determined based on significant changes in membrane potential (mV) and/or firing rate (Hz) and expressed relative to baseline (Δ membrane potential and % frequency change). DRN neurons were classified as sensitive or insensitive to the 5-HT_{1A} agonist 8-OH-DAPT. (A) SEP-856 induced inhibitory responses in the majority of spontaneously active, 8-OH-DAPT-sensitive DRN neurons. This inhibitory effect was absent in 4/5 neurons the presence of the 5-HT_{1A} antagonist WAY-635 (B) but persisted in 5/6 neurons in the presence of the TAAR1 antagonist EPPTB (C). (D) Based on significant changes in membrane potential and/or firing rate, approximately half of the spontaneously-active neurons in the VTA were inhibited by SEP-856 treatment when compared to baseline. This effect persisted in presence of WAY-635 (E, n = 2 neurons) but was reduced in the presence of EPPTB (F, n = 2 neurons). Histograms show neuronal responses following administration of SEP-856 alone (control, A and D) and in the presence of WAY-635 (B and E) or EPPTB (C and F). Solid horizontal bars indicate the timeframe of compound administration to the slice. Two-tailed t-test, * p < 0.05. Data are shown as mean ± s.e.m.

Figure 8. SEP-856 inhibits firing of DRN *in vivo* and partially inhibits PCP-induced hyperactivity through 5-HT_{1A} receptors. (A) SEP-856 (2 mg/kg, i.v.) decreased single-unit discharges of DRN neurons in anesthetized Sprague Dawley rats (n = 2). (B) The most significant inhibition was observed during the first 10 minutes after dosing, with discharge rates returned to baseline levels ~20 minutes later (48-53 minutes after start of recording). (C, D) Neuronal DRN firing rates were completely suppressed by a higher dose of SEP-856 (5 mg/kg, i.v., two-tailed t-test, *p < 0.05, n = 2), and this inhibition was subsequently reversed by WAY-635 (0.08 mg/kg; n = 1). (E) Pretreatment of WAY-635 (1 mg/kg, i.p.) partially attenuated the ability of SEP-856 (3 mg/kg, p.o.) to reduce PCP-induced hyperactivity in C57BL/6J mice (1-way ANOVA + Tukey's post hoc test, +p < 0.05, *p < 0.05 vs. Veh/Veh/PCP, #p < 0.05 vs Veh/Veh/Veh; n = 9-10/group). Data are shown as mean ± s.e.m.

MAIN TABLES

Table1. Receptor affinities.

Panel screens of up to 105 radioligand binding assays and 34 enzyme assays were performed and the affinity of SEP-856 was determined for receptors at which >50% inhibition was seen at 10 μ M. Details of radioligands and incubation conditions are listed in the Material and Methods section and the targets in the screening panels are listed in the Supplemental Information. Data are shown as mean \pm s.e.m. ($n \geq 3$, $n = 1$ for 5-HT_{2B}).

Receptor	Ki (μ M)
D _{2s}	21.3 \pm 8.2
5-HT _{1A}	0.284 \pm 0.056
5-HT _{1B}	1.90 \pm 1.72
5-HT _{1D}	1.13 \pm 0.21
5-HT _{2A}	17.25 \pm 4.0
5-HT _{2B}	1.1
5-HT _{2C}	2.45 \pm 0.82
5-HT ₇	0.031 \pm 0.003
α _{2A}	0.59 \pm 0.06
α _{2B}	1.9 \pm 0.10

Table 2. *In vitro* functional profile of SEP-856.

The functional effects of SEP-856 were determined for receptors at which >50% inhibition was seen in the panel screens. The *in vitro* pharmacology studies were run in both agonist and antagonist modes. The specific assays are listed in the Materials and Methods section. Data are shown as mean \pm s.e.m. (n \geq 3).

Receptor	Agonist		Antagonist	
	EC ₅₀ (μ M)	% Emax	IC ₅₀ (μ M)	% inhibition
TAAR1	0.140 \pm 0.062	101.3 \pm 1.3	NE	NE
5-HT _{1A}	2.3 \pm 1.40	74.7 \pm 19.6	NE	NE
5-HT _{1B}	15.6 \pm 11.60	26.6 @ 10 μ M	NE	NE
5-HT _{1D}	0.262 \pm 0.09	57.1 \pm 6.0	NE	NE
5-HT _{2A}	>10	29.3 @ 10 μ M	NE	NE
5-HT _{2C}	30 \pm 4.5	63.3 \pm 3.1	NE	NE
5-HT ₇	6.7 \pm 1.32	41.0 \pm 9.5	NE	NE
α _{2A}	>10	39.4 \pm 4.2	NE	NE
α _{2B}	NE	NE	NE	NE
D _{2L} (cAMP)	10.44 \pm 4.0	23.9 @ 10 μ M	>100	34.0 \pm 1.16
D _{2L} (arrestin recruitment)	8.02	27.1 @ 10 μ M	>100	<25 @ 100 μ M

NE = no effect (<30% @ 30 μ M)

MAIN FIGURES

Figure 1

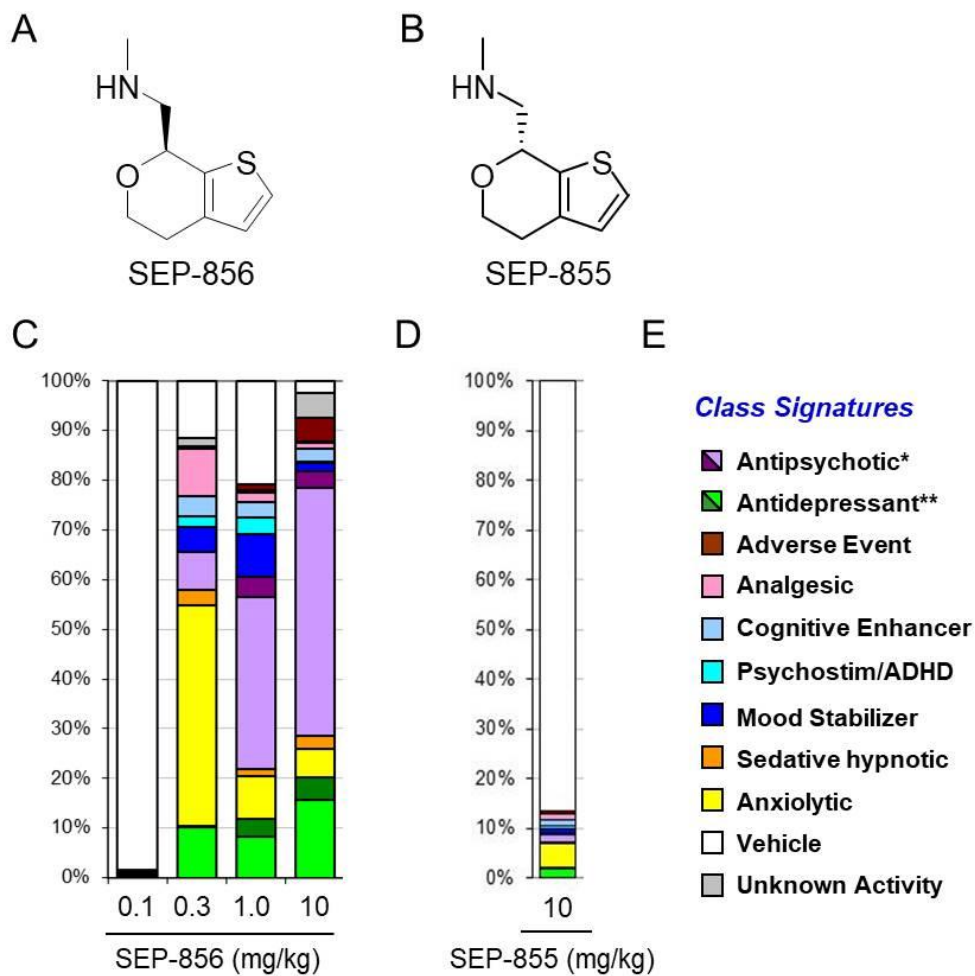


Figure 2

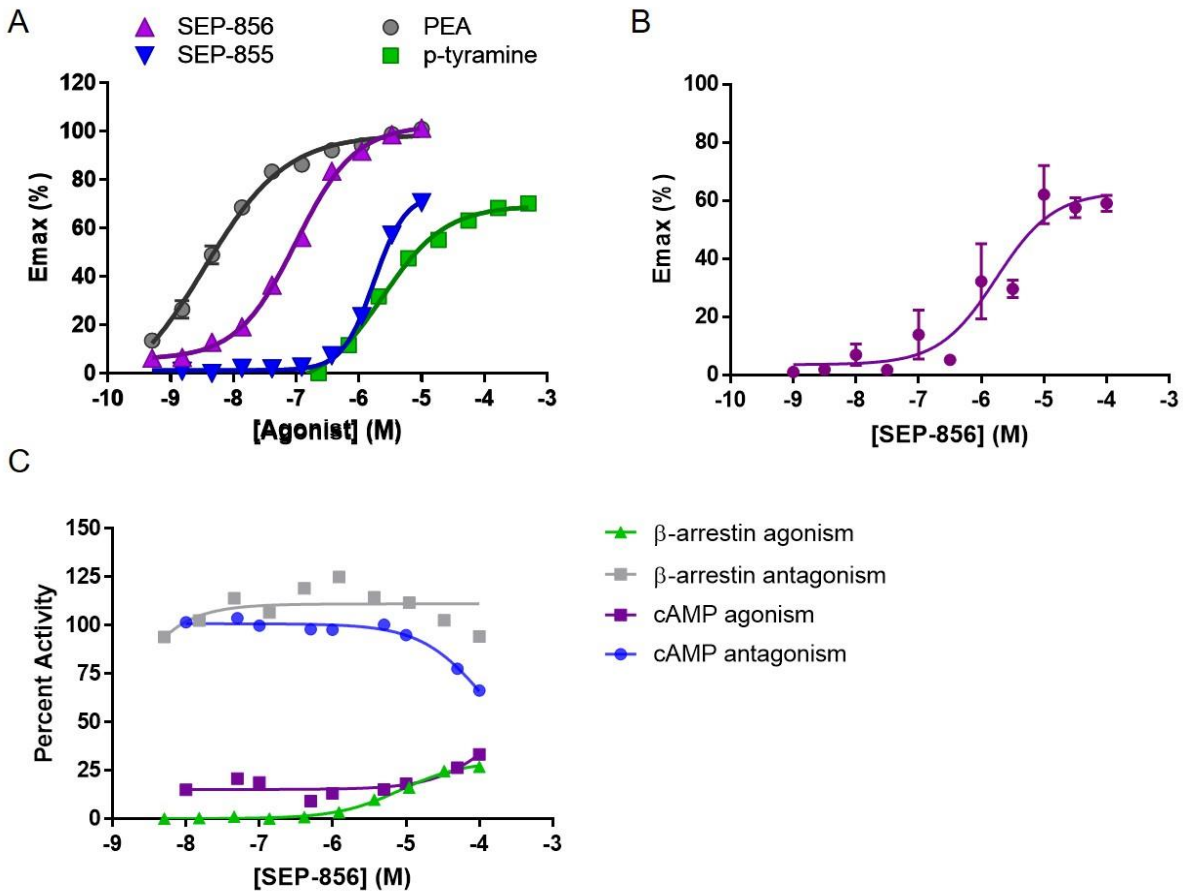


Figure 3

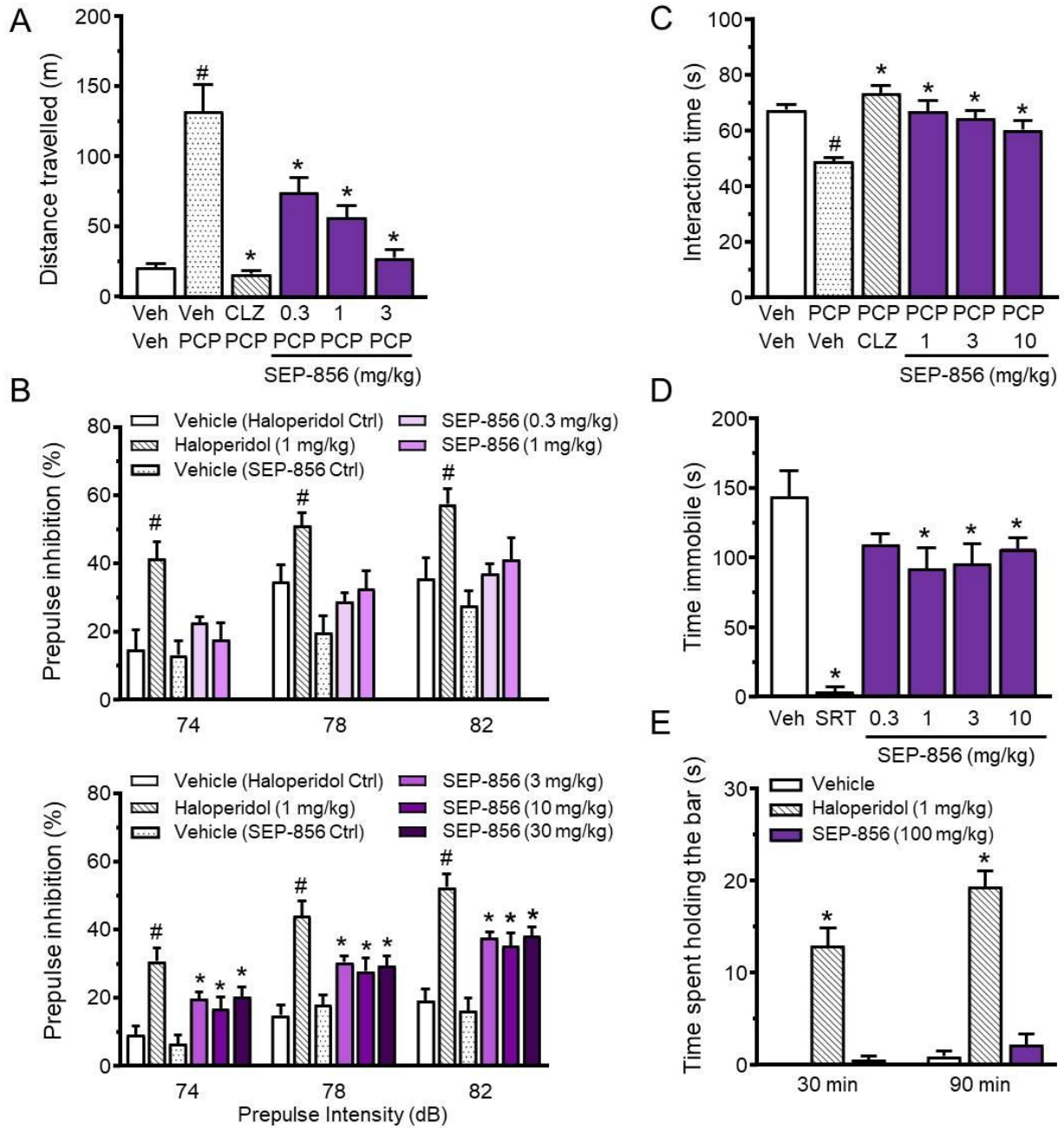


Figure 4

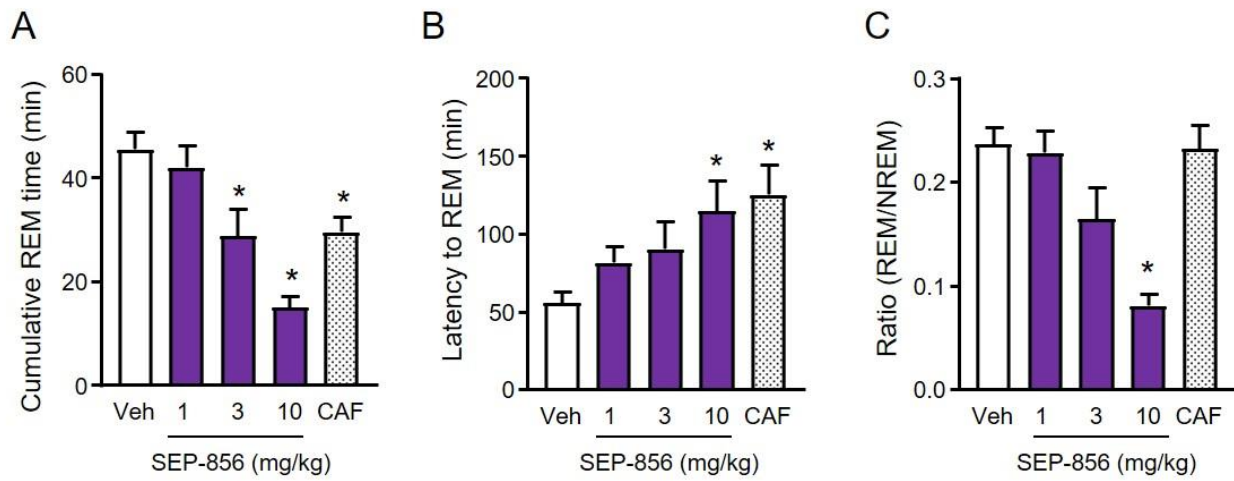
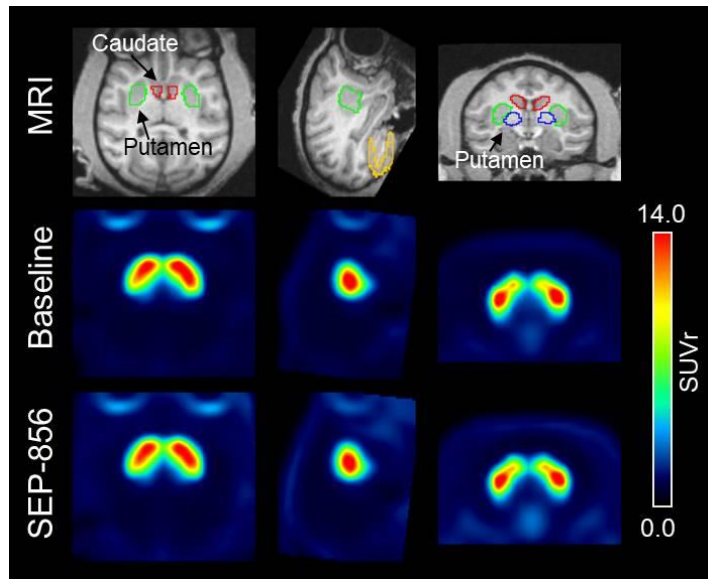
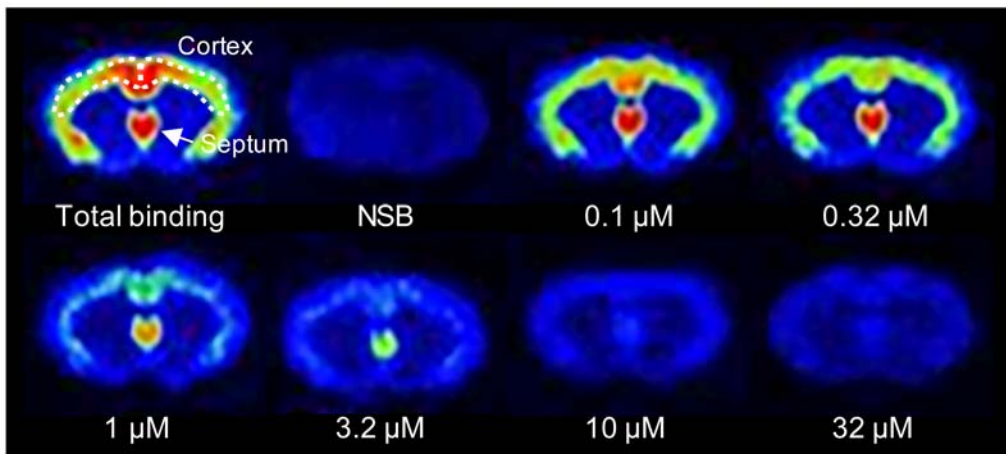


Figure 5



A



B

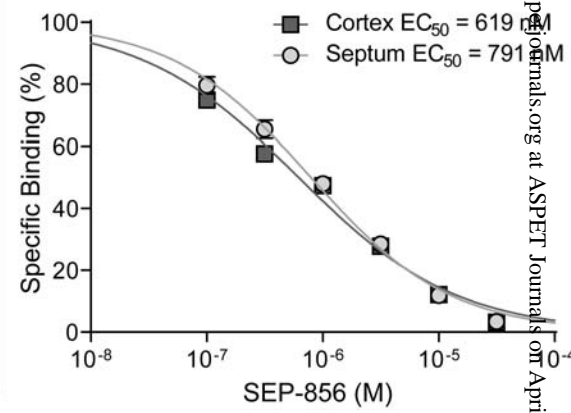


Figure 7

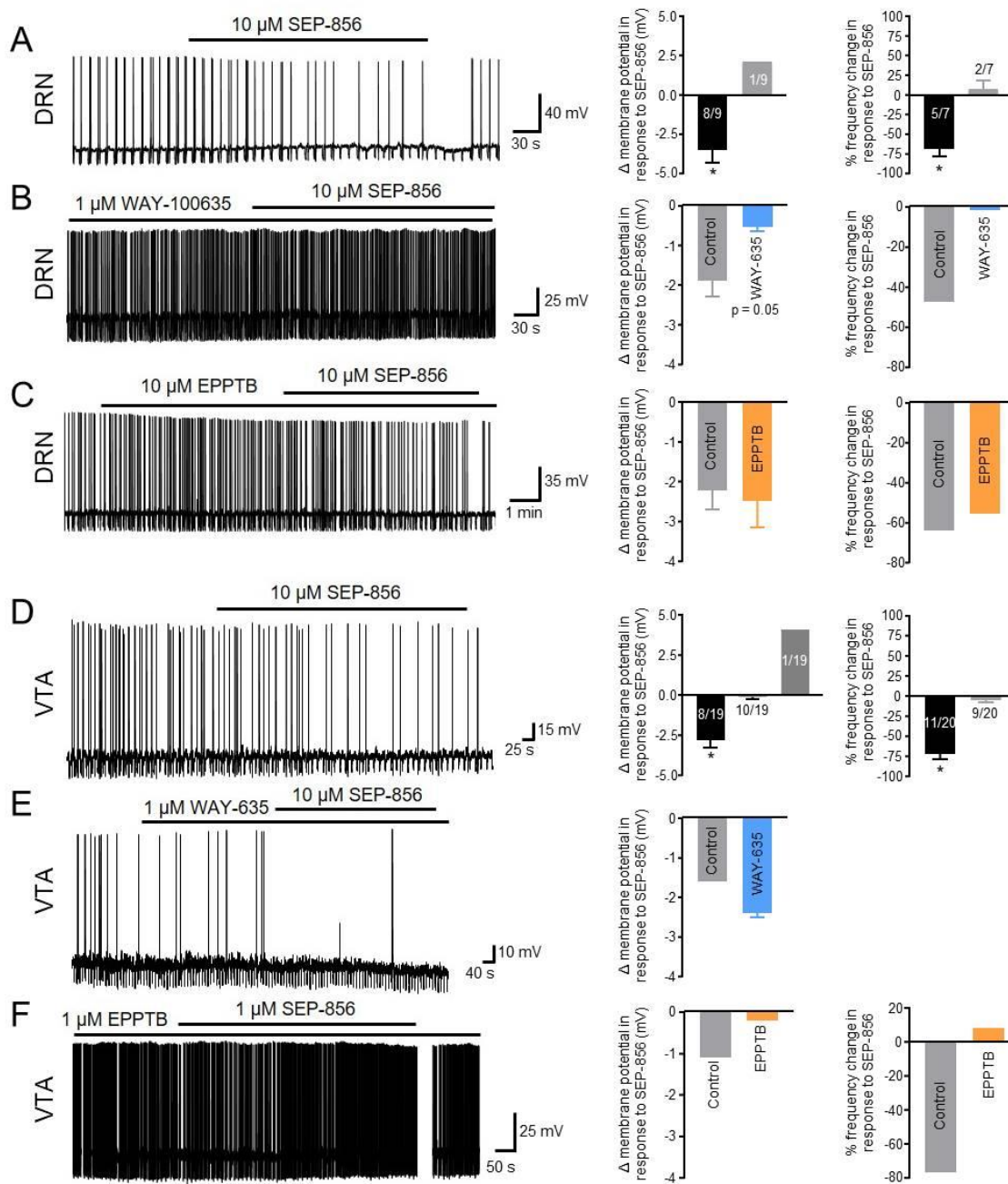


Figure 8

