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The Atypical Dopamine Transporter Inhibitor CE-158 Enhances Dopamine Neurotransmission in the Prefrontal Cortex of Male Rats: A Behavioral, Electrophysiological, and Microdialysis Study

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Abstract

Background: Dopamine plays a key role in several physiological functions such as motor control, learning and memory, and motivation and reward. The atypical dopamine transporter inhibitor S,S stereoisomer of 5-(((S)-((S)-(3-bromophenyl)(phenyl)methyl)sulfinyl)methyl)thiazole (CE-158) has been recently reported to promote behavioral flexibility and restore learning and memory in aged rats.

Methods: Adult male rats were i.p. administered for 1 or 10 days with CE-158 at the dose of 1 or 10 mg/kg and tested for extracellular dopamine in the medial prefrontal cortex by means of intracerebral microdialysis and single unit cell recording in the same brain area. Moreover, the effects of acute and chronic CE-158 on exploratory behavior, locomotor activity, prepulse inhibition, working memory, and behavioral flexibility were also investigated.

Results: CE-158 dose-dependently potentiated dopamine neurotransmission in the medial prefrontal cortex as assessed by intracerebral microdialysis. Moreover, repeated exposure to CE-158 at 1 mg/kg was sufficient to increase the number of active pyramidal neurons and their firing frequency in the same brain area. In addition, CE-158 at the dose of 10 mg/kg stimulates exploratory behavior to the same extent after acute or chronic treatment. Noteworthy, the chronic treatment at both doses did not induce any behavioral alterations suggestive of abuse potential (e.g., motor behavioral sensitization) or pro-psychotic-like effects such as disruption of sensorimotor gating or impairments in working memory and behavioral flexibility as measured by prepulse inhibition and Y maze.

Conclusions: Altogether, these findings confirm CE-158 as a promising pro-cognitive agent and contribute to assessing its preclinical safety profile in a chronic administration regimen for further translational testing.

Keywords: ADHD, chronic treatment, cognitive enhancer, dopamine transporter, prefrontal cortex

Significance Statement

The atypical dopamine transporter inhibitor S,S stereoisomer of 5-(((S)-((S)-(3-bromophenyl)(phenyl)methyl)sulfinyl)methyl)thiazole (CE-158) has been recently synthesized and characterized by our laboratories, evidencing promising functional and behavioral effects after systemic acute administration. However, a comprehensive understanding of key features (and possible side effects) associated with a chronic treatment with CE-158 is critical toward a therapeutic strategy based on the enhancement of dopamine neurotransmission via dopamine transporter inhibition. Indeed, psychostimulants are well recognized for their pro-psychotic consequences or abuse liability, making them unsuitable for clinical approval. We present here an investigation in adult male rats repeatedly administered with CE-158 at different doses. Under our conditions, CE-158 proved no evidence of inducing psychotic signs or abuse liability, whereby it corroborated its prefrontal-related pro-active efficacy. Our findings support the safety profile of CE-158 as a promising agent for the treatment of pathological conditions, such as attention deficit hyperactivity disorder or dementia, characterized by altered prefrontal dopamine neurotransmission.

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INTRODUCTION

Dopamine plays a key role in several physiological functions such as motor control, reproductive behaviors, learning and memory, and motivation and reward (Beaulieu and Gainetdinov, 2011; Klein et al., 2019; Melis et al., 2022). The central role of dopamine in these functions is highlighted by the fact that alterations in dopamine neurotransmission lead to several neurological and/or neuropsychological disturbances, such as Parkinson disease, schizophrenia, mood disorders, attention deficit hyperactivity disorder (ADHD), and substance use disorders (Castellanos and Tannock, 2002; Nestler et al., 2002; Maia and Frank, 2017; Volkow et al., 2017; de Natale et al., 2018).

In this regard, the membrane dopamine transporter (DAT) represents a privileged target for the development of new classes of drugs (German et al., 2015), being a key factor in regulating synaptic dopamine in both physiological and pathological contexts (Kuhar et al., 1990; Gainetdinov and Caron, 2003; Leo et al., 2018; Sanna et al., 2020). Accordingly, the atypical DAT inhibitor modafinil has been approved for the treatment of sleeping disturbances and narcolepsia. Modafinil has also been proposed for the treatment of neurological and psychiatric conditions (e.g., Alzheimer disease or ADHD) as well as drug addiction (Mereu et al., 2013) and also as a cognitive enhancer in healthy people (Repantis et al., 2010; Battleday and Brem, 2015). However, in this latter case, there are some important ethical considerations (Brühl et al., 2019). Although it shares a common mechanism of action with other psychostimulants such as cocaine and methamphetamine, modafinil has conversely shown low abuse liability (Wood et al., 2014; Bisagno et al., 2016) due to its reduced interference with mesolimbic dopamine. Accordingly, preclinical studies with modafinil and modafinil analogues displayed an improved circuit-related selectivity of these new classes of compounds (Mereu et al., 2017; Sagheddu et al., 2020).

Among novel modafinil analogues, CE-123 has been found effective in enhancing the motivational tone (Rotolo et al., 2019), increasing memory performance (Kristofova et al., 2018; Lubec et al., 2023), and improving behavioral flexibility and impulsivity control (Nikiforuk et al., 2017), with low abuse liability (Sagheddu et al., 2020). Similarly, a high-affinity analogue, S,S stereoisomer of 5-(((S)-((S)-(3-bromophenyl)(phenyl)methyl)sulfinyl)methyl)thiazole (CE-158), demonstrated the ability to increase motivational tone in a model of impaired effort-related behavior, improve social memory and behavioral flexibility, and reinstate hippocampal synaptic plasticity in the aging brain (Kalaba et al., 2020; Rotolo et al., 2020; Lubec et al., 2021; Ebner et al., 2022).

The prefrontal cortex (PFC) represents a brain area of particular interest for the actions of these compounds as here dopamine is directly involved in higher-order cognitive processes and executive functions (Robbins and Arnsten, 2009; Spencer et al., 2015) by acting on different neuronal populations, in particular pyramidal neurons and GABAergic interneurons, through local circuits and projections to other cortical and subcortical brain regions (Anastasiades and Carter, 2021; Howland et al., 2022). Dysfunctions at these networks are at the basis of peculiar symptoms of pathological conditions such as ADHD, schizophrenia, dementia, drug addiction, and a state often referred to as “hypofrontality.” Hypofrontality is characterized by impulsive-compulsive behaviors and impairments in working memory and attentive and preattentive processes such as sensorimotor gating, stereotypies, and perseverations.

The present study aims at (1) investigating the ability of CE-158 to potentiate dopamine neurotransmission at the level of the

PFC by means of intracerebral microdialysis and in vivo electrophysiology; and (2) investigating the behavioral effects of CE-158 after acute and chronic treatment on PFC-related behaviors, with particular attention to potential side effects due to the chronic administration.

METHODS

Subjects

Male Sprague-Dawley rats (Harlan Nossan, San Pietro al Natisone, Italy), weighing 250–300 g, were kept in groups of 4 in standard cages under controlled environmental conditions (22°C ± 2°C, 60% humidity, 12-hour-light/-dark cycle, with lights on from 7:00 AM to 7:00 PM), with water and standard laboratory food ad libitum. All the experiments were performed between 10:00 AM and 6:00 PM according to the guidelines of the European Communities Directive (2010/63/EU) and the Italian Legislation (D.P.R. 116/92) and were approved by the Ethical Committee for Animal Experimentation of the University of Cagliari.

Drugs

CE-158 was synthesized as previously described (Lubec et al., 2021). Kolliphor EL was purchased from Sigma Aldrich (Sigma-Aldrich Chemie GmbH, Germany). All the other reagents were from commercial sources and of the highest purity available.

Experimental Design

According to the 3R principles governing animal experimentation, all possible effort was made to minimize animal suffering and reduce the number of animals used. Four cohorts of adult male rats were divided in 3 treatment groups: controls (30% Kolliphor EL in physiological solution, 1 mL/kg), 1 mg/kg mg/kg CE-158, and 10 mg/kg CE-158. Rats were administered i.p. once a day for 10 consecutive days according to their treatment group. The first cohort was used for the microdialysis experiments that were performed at the 10th day of treatment as described below. The second cohort was used to perform the electrophysiology experiments. In this case, on the last day of treatment, one-half of them, belonging to the 3 treatment groups, were directly used within 45–60 minutes after the last administration for the electrophysiological recordings into the mPFC. The second half was used for dose-curve experiments, and in this case incremental doses of CE-158 were injected i.v. to 10 mg/mL/kg cumulative dose during the electrophysiology recordings. The third cohort was used to perform the behavioral experiments on exploration, locomotion, and grooming that were performed in the same animals after the first and last treatments at day 1 and day 10, respectively. Finally, the fourth cohort was devoted to the Y-maze and PPI experiments that were performed in the same animals after the first (day 1) and last (day 10) treatments. Regarding behavioral studies and according to the microdialysis results, rats received the i.p. treatment with CE-158 or vehicle 30–45 minutes before the beginning of the test.

Microdialysis Experiment for Determination of Extracellular Dopamine in mPFC

The intracerebral microdialysis for the determination of dopamine content in the mPFC of rats treated with CE-158 or vehicle was performed in awake, freely moving animals as already described (Sanna et al., 2015, 2017). The day before the experiment, rats were positioned in a stereotaxic apparatus (Stoelting

Co., Wood Dale, IL, USA) and, under isoflurane anesthesia (1.5%–2%) (Harvard Apparatus, Holliston, MA, USA), were implanted with a vertical microdialysis probe with a dialysis membrane of approximately 2–3 mm of free surface directed unilaterally at the mPFC (PrL and IL; coordinates: 3.0 mm anterior and 0.7 mm lateral to bregma, and 5.5 mm ventral to dura) according to Paxinos and Watson (2004). The day of the experiment, the animals were transferred to a sound-proof room and after a 1-hour habituation period, the microdialysis probe was connected with polyethylene tubing to a CMA/100 micro-infusion pump (Harvard Apparatus, Holliston, MA, USA) and perfused with a Ringer's solution (147 mM NaCl, 3 mM KCl, and 1.2 mM CaCl₂, pH 6.5) at a flow rate of 2.5 µL/min. After an equilibration period of 2 hours of the perfusion medium with the extracellular fluid, dialysate aliquots of 37.5 µL were collected every 15 minutes in polyethylene tubes kept on ice. After the collection of 4 aliquots, rats were administered i.p. with 1 or 10 mg/kg CE-158 or vehicle, and other 8 dialysate aliquots were collected every 15 minutes. Dopamine concentrations in the dialysates were measured by high-pressure liquid chromatography on a 7.5-cm × 3.0-mm i.d., Supelcosil C18, 3-µm-particle size column (Supelco, Supelchem, Milan, Italy) coupled to electrochemical detection (Coulchem II, ESA, Cambridge, MA, USA) using a 5011 dual cell. Detection was performed in reduction mode, with potentials set to +350 and –180 mV. The mobile phase was 0.06 M citrate/acetate pH 4.2, containing methanol 20% v/v, 0.1 mM EDTA, 1 µM triethylamine, and 0.03 mM sodium dodecyl sulphate at a flow rate of 0.6 mL/min. The sensitivity of the assay was 0.125 pg. For the procedures related to the histological verification of probe placement, see [supplementary Figure 1](#).

In Vivo Single-Unit Electrophysiological Recordings

Rats were anesthetized with 400 mg/kg i.p. chloral hydrate and placed in the stereotaxic frame. Single neurons located in layers III–VI of prelimbic/infralimbic cortex (AP: +2.8–3.6 mm from bregma, L: 0.8–1.0 mm from the midline, V: 1.5–4.0 mm from the cortex) was recorded with glass micropipettes filled with 0.5 M sodium acetate. Individual [action potentials](#) were isolated and amplified (1- to 10,000-Hz filter) by means of a CP511AC amplifier (Grass Instruments Co., Quincy, MA, US). Experiments were sampled with Spike2 software by a CED1401 interface (Cambridge Electronic Design, Cambridge, UK). Two types of neurons were isolated and recorded based on waveform shape and frequency of action potentials. Putative pyramidal neurons were selected in accordance with biphasic positive–negative deflections and >2-ms-wide action potentials, regular or irregular activity (Connors and Gutnick, 1990; Au-Young et al., 1999; Secci et al., 2019). Putative GABA-interneurons were identified according to biphasic negative–positive deflections in accordance with single-unit recordings from other brain regions (Steffensen et al., 1998; Schwaller et al., 2004). In such experimental settings, all types of neurons exhibited a <10-Hz action potential discharge rate. To estimate the general activity, the electrode was passed in 4–6 tracks, and the number of active cells was divided by the number of tracks. Spontaneous firing rate and firing regularity (expressed by coefficient of variation, CV, the SD of interspike intervals divided by the mean interspike interval) were determined.

For dose-curve experiments, CE-158-induced changes in firing frequency were calculated by averaging the effects for the 2-minute period following acute i.v. administration of each dose.

Locomotor Activity, Rearing, and Grooming

The day before the first experiment session (i.e., the day before acute treatment) rats underwent two 1-hour habituation sessions to prevent the influence of novelty factors linked to the experimental procedure and motility apparatus (Angioni et al., 2016). To this aim, rats were transferred to a soundproof room with a light level of 30 lux and positioned in individual cages for habituation. The day of the experiment, rats were transferred in the same experimental room and, after 1 hour, CE-158 or vehicle was administered. Rats were individually tested with a Digiscan Animal Activity Analyzer (Omnitech Electronics, Columbus, OH, USA). Each cage (42 cm × 42 cm × 63 cm) had 2 sets of 16 photocells located at right angles to each other, projecting horizontal infrared beams 2.5 cm apart and 2 cm above the cage floor. Horizontal activity was measured from 30 to 90 minutes after the treatment (for a total of 60 minutes) as total number of sequential infrared beam breaks (counts) in the sensors, recorded every 5 minutes, beginning immediately after placing the animals into the cage. Rearing and grooming episodes were counted by 2 independent observers unaware of the treatment conducted on videotape recordings. Rearing episodes were counted when the rat stretched vertically on its hind legs and exhibited the classic sniffing behavior of the surrounding environment. Grooming behavior was defined as reported in [Berridge and colleagues \(2005\)](#).

PPI of the Startle Response

Rats were tested as previously described (Noli et al., 2017; Sagheddu et al., 2021). The apparatus (Med Associates) consisted of 4 standard cages placed in sound-attenuated chambers with fan ventilation. Each cage consisted of a Plexiglas cylinder (5 cm diameter) mounted on a piezoelectric accelerometric platform connected to an analog-digital converter. Two separate speakers conveyed background noise and acoustic bursts, each one properly placed to produce a variation of sound within 1 dB across the startle cage. Before each testing session, acoustic stimuli and mechanical responses were calibrated. The testing session featured a background noise of 70 dB and consisted of an acclimatization period of 5 minutes, followed by 3 consecutive sequences of trials (blocks). Unlike the first and the third block, during which rats were presented with only 5 pulse-alone trials of 130 dB, the second block consisted of a pseudorandom sequence of 50 trials, including 12 pulse-alone trials; 30 trials of pulse preceded by 68-, 70-, or 80-dB prepulses (10 for each level of prepulse loudness); and 8 no-stimulus trials, where only the background noise was delivered. Inter-trial intervals were randomly selected between 10 and 15 seconds. The percent PPI value was calculated using the following formula: $100 - [(mean\ startle\ amplitude\ for\ prepulse\ pulse\ trials / mean\ startle\ amplitude\ for\ pulse\ alone\ trials) * 100]$. For the analyses of data presented here, PPI values related to different prepulse intensities were collapsed, given that no significant differences among them were observed.

Y-Maze

On the day of the test, rats were transferred to a soundproof room and left in their home cages for 30 minutes for habituation. Then they were treated with CE-158 or vehicle and after 30 minutes positioned in a Y-maze. Rats were randomly placed at the end of 1 arm of the Y-maze, and the sequence of arm entries was recorded for 10 minutes. The number and sequence of arm entries referred to the 10-minute test were counted for each rat and analyzed by 2 independent observers unaware of the treatment done

on videotape recordings. An arm visit was recorded when a rat moved all 4 paws into the arm. An alternation was defined as consecutive entries into all 3 arms (e.g., 1, 2, 3 or 1, 3, 2). The number of maximum alternations was the total number of arm entries minus 2, and the percentage of alternations was calculated as the ratio of actual to maximum alternations multiplied by 100: (actual alternations/maximum alternations)*100 (see for instance, Carton et al., 2021).

Data Analysis and Statistics

All data are given as mean \pm SEM and are expressed as absolute values or percentages.

For in vivo electrophysiology, statistical significance was assessed using parametric 1-way ANOVA. Contingency of population was analyzed by chi-squared test.

For in vivo microdialysis, raw data were percent transformed (with 100% as the average of the last 4 dopamine basal values before treatment), and statistical analyses were performed with 2-way repeated-measures (RM)-ANOVAs using treatment as the between-subjects factor and time (i.e., dialysate fractions) as the within-subjects factor.

For the behavioral tests, 2-way RM-ANOVAs using treatment as the between-subjects factor and time (i.e., duration of treatment or time fraction during the experiment) as the within-subjects factor were performed.

When ANOVA revealed statistically significant main effects or interactions, pairwise comparisons were performed by using Bonferroni corrected paired *t* tests or the Tukey multiple comparison test, respectively.

All analyses were performed by using the software GraphPad Prism 8. The significance level was set at $P < .05$.

RESULTS

Microdialysis in mPFC for Determination of Extracellular Dopamine

The first goal of our investigation was to assess the ability of chronic treatment with CE-158 to stimulate dopamine neurotransmission in the mPFC, similar to that seen in other brain areas (Lubec et al., 2021; Ebner et al., 2022). To this aim, we measured extracellular dopamine levels in the mPFC by means of intracerebral microdialysis following systemic administration of 1 or 10 mg/kg CE-158. Basal extracellular dopamine amounts in the mPFC did not differ among the 3 treatment groups and were approximately 2.20 pg in 20 μ L of dialysate, corresponding to an extracellular dopamine concentration of approximately 0.70 nM. As shown in Figure 1, CE-158 produced a dose-dependent increase in extracellular dopamine concentrations up to 30% and 70% above basal values, for 1 and 10 mg/kg, respectively (2-way ANOVA, dose*time interaction: $F_{(22,132)} = 2.73$; dose: $F_{(2,12)} = 14.16$; both $P < .001$). Moreover, Tukey post hoc comparisons indicated that although CE-158 is effective in increasing dopamine content in the mPFC already 15 minutes after the treatment, the peak is detectable at 30 minutes, with the effect lasting for at least 75–105 minutes (see Figure 1 for single points of statistical significance).

In Vivo Single-Unit Extracellular Recordings From the mPFC

We previously showed that acute administration of CE-158 activates in vivo pyramidal neurons from the mPFC of young and old rats (Lubec et al. 2021). Here, we extended the functional analysis of the electrophysiological properties of putative pyramidal

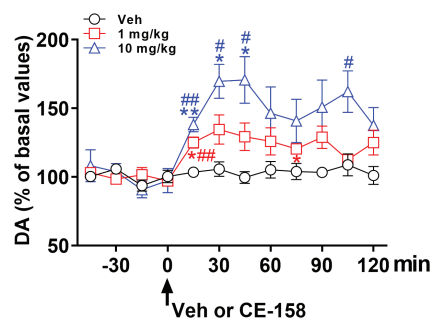


Figure 1. Extracellular dopamine (DA) concentrations in the medial prefrontal cortex (mPFC) dialysates obtained from rats treated with CE-158 (1 or 10 mg/kg, i.p.) or vehicle (Veh). Data are reported as percentages (with 100% as the average of the last 4 dopamine basal values before treatment) with values expressed as means \pm SEM of 5 rats per group. * $P < .05$ compared with basal values (before treatment); # $P < .05$ with respect to vehicle-treated rats (2-way RM-ANOVA followed by Tukey multiple-comparison test).

neurons (Figure 2A, top left) and GABA interneurons (Figure 2A, bottom left) following chronic treatment with CE-158 for 10 days or vehicle as control. We recorded 50 pyramidal and 30 GABA cells from controls ($n = 9$ rats), 66 pyramidal and 27 GABA cells from rats treated with 1 mg/kg ($n = 9$), and 49 pyramidal and 35 GABA cells from rats treated with 10 mg/kg ($n = 9$). A χ^2 test revealed that CE-158 did not change the population of active neurons among groups ($\chi^2_{(2)} = 3.41$, $P = .18$; Figure 2A, right).

Chronic treatment with 1 mg/kg CE-158 increased the number of spontaneously active cells (3.12 ± 0.46 cells per track) (Figure 2B; 1-way ANOVA $F_{(2,24)} = 3.6$; $P = .043$) compared with control treatment (1.837 ± 0.271 cells per track) and with high-dose treatment (1.942 ± 0.373 cells per track).

Moreover, analysis of firing activity showed an increased firing frequency (Figure 2C; 1-way ANOVA $F_{(2,161)} = 4.719$; $P = .010$) by CE-158 1 mg/kg (2.59 ± 0.29 Hz; ctrl: 1.52 ± 0.19 Hz; 10 mg/kg: 2.03 ± 0.23 Hz) but no change in the pattern as measured by the coefficient of variation (Figure 2D; 1-way ANOVA $F_{(2,161)} = 0.613$; $P = .543$) in the 3 groups (ctrl: $130.5 \pm 4.72\%$; 1 mg/kg: $130.6 \pm 3.92\%$; 10 mg/kg: $136.8 \pm 4.9\%$).

Neither the number of spontaneously active interneurons (Figure 2E; 1-way ANOVA $F_{(2,24)} = 0.098$, $P = .91$) nor the firing frequency (Figure 2F; 1-way ANOVA $F_{(2,89)} = 0.33$, $P = .72$) or pattern (Figure 2G; 1-way ANOVA $F_{(2,89)} = 0.017$, $P = .98$) was altered by chronic treatment with 1 mg/kg CE-158 (firing 3.69 ± 0.49 Hz, CV $125.7 \pm 4.16\%$) or 10 mg/kg CE-158 (firing 3.19 ± 0.49 Hz, CV $124.8 \pm 4.41\%$) compared with its vehicle (firing 3.22 ± 0.46 Hz, CV $125.9 \pm 4.50\%$).

Cumulative dose-response curves to CE-158 (1.25–10 mg/kg i.v.) were also performed to compare its effect on pyramidal neuron activity at the end of the 10 days of chronic exposure. In vehicle-pretreated rats, acute administration of CE-158 elicited a plateau in firing rate (Figure 3A), while in rats pretreated with the lower dose (Figure 3B) the frequency significantly augmented at the maximal cumulative dose (RM 2-way ANOVA, $F_{(4,48)} = 3.57$, $P = .012$). Finally, in rats pretreated with the higher dose (Figure 3C), acute injection of CE-158 increased pyramidal neuron firing in a dose-dependent fashion.

Exploratory Behavior

Exploratory behaviors are widely acknowledged as suggestive of general well-being in rodents, whereas diminished locomotion

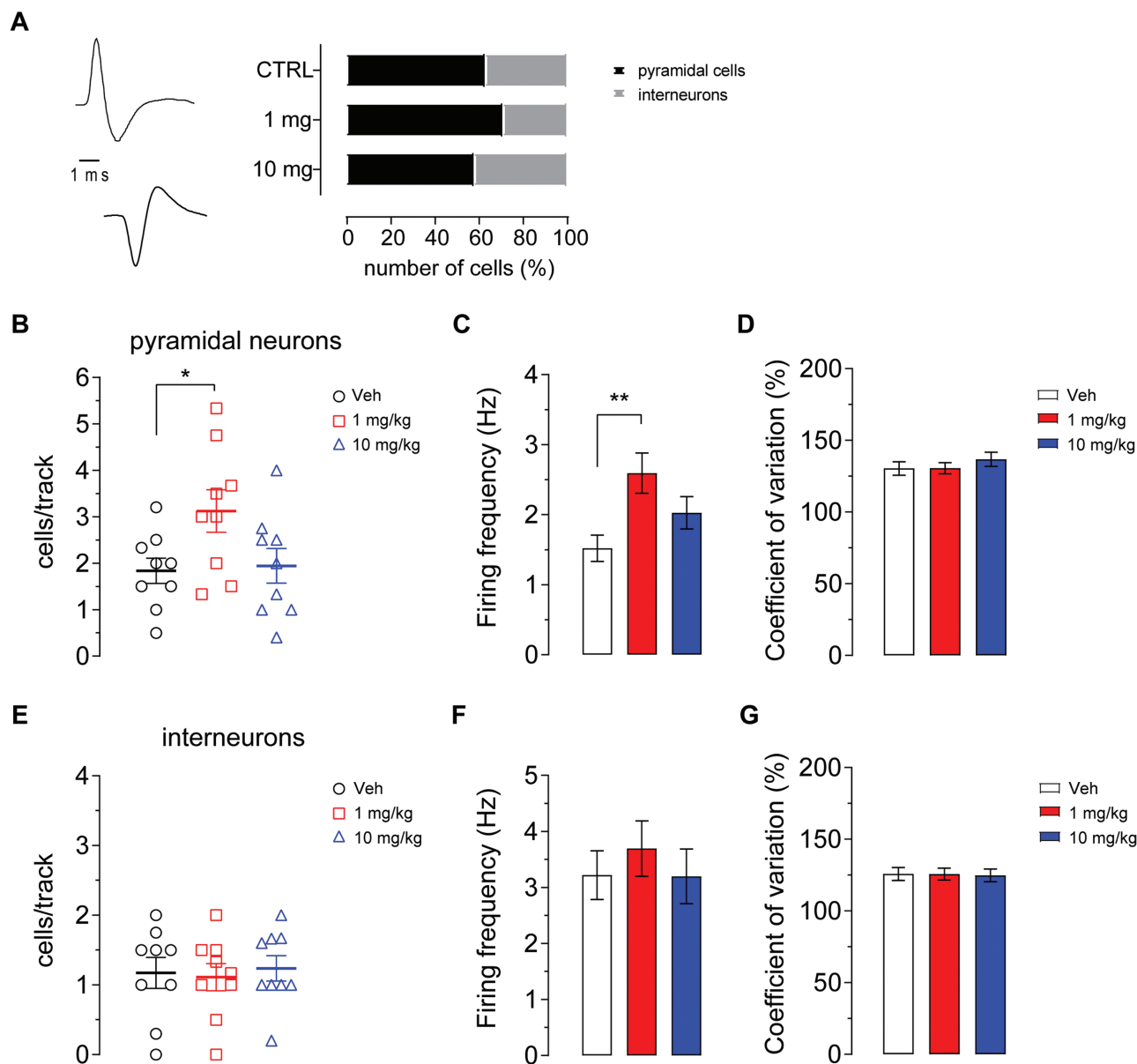


Figure 2. Typical action potential of pyramidal neuron (A, top) and γ -aminobutyric acid (GABA) interneuron (A, bottom) extracellularly recorded from the medial prefrontal cortex (mPFC). Histograms showing the percentage of pyramidal neurons and GABA interneurons from the mPFC of the 3 experimental groups (A, right). Number of mean active pyramidal neurons from the mPFC of the 3 experimental groups (B). Mean firing frequency (C) and coefficient of variation (D) of pyramidal neurons from the mPFC of the 3 experimental groups. Number of mean active interneurons from the mPFC of the 3 experimental groups (E). Mean firing frequency (F) and coefficient of variation (G) of interneurons from the mPFC of the 3 experimental groups. Values are expressed as mean \pm SEM. * $P < .05$; ** $P < .01$ compared with vehicle (Veh)-treated rats (1-way ANOVA followed by Bonferroni multiple comparison test).

and rearing have been associated with stress and discomfort (Pisula and Siegel, 2005; Sturman et al., 2018). Accordingly, typical exploratory and interactive behaviors could be affected by dopamine dysregulations, which may result from both pathological conditions and disruptive drugs (Arenas et al., 2016). As reported in Figure 4, CE-158 dose-dependently increased the number of rearing episodes to the same extent after the first (i.e., acute) and the last drug administration (i.e., after 10 days of treatment). The number of rearings, although with some differences between the 2 doses, was higher during the first 30 minutes after the positioning of the animal into the experimental cage and tended to decrease towards control values in the second 30 minutes, both at day 1 (Figure 4A) and day 10 (Figure 4B) of treatment. Accordingly, RM 2-way ANOVA detected a significant effect of

time ($F_{(4,14,5,53)} = 5.533$, $P < .001$) and a significant time*treatment interaction ($F_{(22,143)} = 2.932$, $P < .0001$) for day 1 of treatment and a significant effect of time ($F_{(3,89,5,55)} = 5.55$, $P < .001$) and of treatment ($F_{(2,13)} = 4.323$, $P < .05$) for the test performed after the last treatment at day 10. The efficacy of the compound in stimulating the exploratory behavior after chronic treatment was also confirmed by the analyses performed on the total number of rearing episodes at day 1 and day 10 of treatment (Figure 4C). Accordingly, 2-way ANOVA detected a significant effect of the treatment ($F_{(2,13)} = 9.527$, $P < .01$) but not of the day or a significant day*treatment interaction. Moreover, post hoc comparisons revealed that on both day 1 and 10 of treatment, the rats treated with the dose of 10 mg/kg CE-158, but not those with 1 mg/kg, displayed a significantly higher number of rearing episodes compared

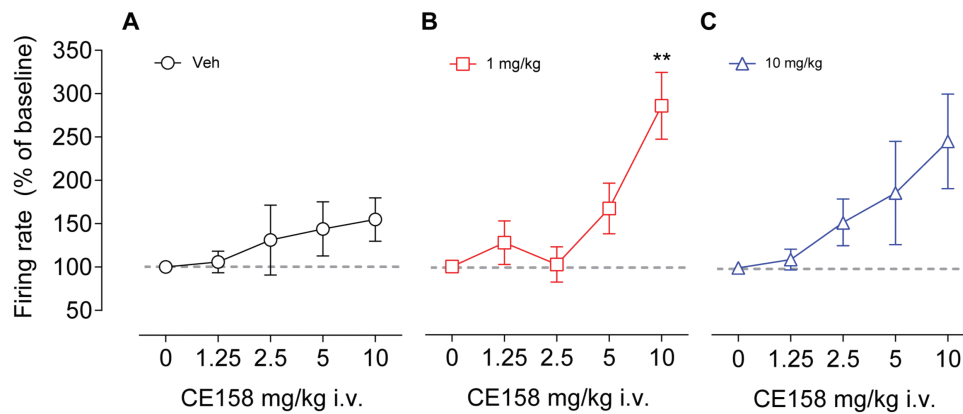


Figure 3. Mean firing rate of medial prefrontal cortex (mPFC) pyramidal neurons following cumulative doses of CE-158 intravenously (i.v.) administered in rats pretreated for 10 days with vehicle (Veh) (A), 1 mg/kg (B), or 10 mg/kg (C). Values are expressed as mean \pm SEM. ** $P < .01$ compared with the matched dose of the vehicle-pretreated rats (RM 2-way ANOVA followed by Bonferroni multiple comparison test).

with vehicle-treated rats. Taken together, these results indicate that CE-158 dose- and time-dependently induces an increase in exploratory behavior and that this effect is maintained without significant changes over a period of chronic treatment of 10 days.

Chronic treatment with psychostimulants can induce exaggerated locomotor activity and behavioral sensitization, which is considered a sign of the addictive effects of a drug (Everitt and Wolf, 2002). Hence, we also investigated if the chronic administration regimen of CE-158 used here was able to induce this effect. To this aim, we measured horizontal locomotor activity in cages equipped with infrared motion detectors. As expected, and similarly to what was seen with rearings (see above), over the test, rats reduced horizontal movements (time, day 1: $F_{(2,19,28,49)} = 48.71$, $P < .0001$; Figure 4D; day 10: $F_{(3,94,51,17)} = 91.64$, $P < .0001$; Figure 4E) but in this case with no difference among treatment groups at both day 1 (treatment: $F_{(2,143)} = 1.073$, $P = .382$; Figure 4D) and day 10 (treatment: $F_{(2,143)} = 1.145$, $P = .307$; Figure 4E). Furthermore, no difference was observed in the total locomotor activity referred to the entire 60-minute test among the 3 treatment groups, at both day 1 and day 10 (2-way ANOVA, $F_{(2,13)} = 0.684$, $P = .52$; Figure 4F), although a general increase in the total counts was observed in the 3 treatment groups passing from day 1 to day 10, regardless the treatment received (2-way ANOVA, main effect of day: $F_{(1,13)} = 7.48$, $P < .05$; Figure 4F). These results indicate that subchronic treatment with CE-158 at 1 and 10 mg/kg does not induce behavioral sensitization, in contrast to classical psychostimulants such as cocaine and amphetamine (Everitt and Wolf, 2002).

PPI

Dopaminergic psychostimulants can solicit abnormalities in sensorimotor gating relevant to psychotic events and schizophrenia (Lapworth et al., 2009; Fiorentini et al., 2021). To exclude possible pro-psychotic side effects of the novel compound CE-158, we performed the PPI of the acoustic startle reflex test, a reliable tool for the assessment of sensorimotor gating acknowledged for high translational significance (Geyer et al., 2001; Mena et al., 2016), as a probe of proper PFC functioning under chronic dopamine stimulation by CE-158 (Shoemaker et al., 2005; Mena et al., 2021).

As reported in Figure 5, neither acute nor chronic treatment of rats with 1 or 10 mg/kg CE-158 affected PPI values (2-way ANOVA, $F_{(2,21)} = 0.175$, $P = .84$) compared with vehicle-treated controls. Similarly to locomotor activity, a slightly significant increase in PPI values was observed for all 3 groups regardless of the treatment received passing from day 1 to day 10 (2-way ANOVA, $F_{(1,21)} = 7.14$,

$P < .05$). Seemingly, CE-158 at the tested doses and over a period of 10 days of treatment did not induce impairment or disruption of sensorimotor gating as potential anticipation towards psychosis.

Y-Maze

We also tested the impact of protracted treatment with CE-158 on working memory and behavioral flexibility, 2 dopamine-related executive functions that are altered in several psychopathological conditions, from psychosis to drug abuse (Waltz, 2017; Potvin et al., 2018), and whose alteration represents a classical sign of hypofrontality (i.e., a condition characterized by impulsive-compulsive behaviors, impairments in working memory and behavioral flexibility, stereotypies, and perseverations). We took advantage of the Y-maze test, a mPFC-related behavioral procedure. As reported in Figure 6A, CE-158 given to rats at the doses of 1 or 10 mg/kg, either after single or 10 days of repeated administration, did not affect the alternance of arm entries compared with vehicle (2-way ANOVA, treatment $F_{(2,21)} = 1.079$, $P = .358$). However, post hoc comparisons revealed a significant difference between vehicle- and 10-mg/kg CE-158-treated rats ($P < .05$) in the alternance index after repeated exposure, although with no difference between acute and chronic administration for both treatment groups. Similarly, as reported in Figure 6B, the total number of arm entries did not differ between the 3 treatment groups on either day 1 or after 10 days of treatment (2-way ANOVA, $F_{(2,21)} = 0.277$, $P = .761$), further confirming that CE-158 does not impair or alter general locomotion/exploration in rats. The results obtained with the Y-maze suggest that a 10-day chronic treatment with CE-158 does not alter or disrupt exploration, flexibility, and/or working memory.

Grooming

Excessive grooming is considered a putative sign of stereotyped and compulsive behavior (Berridge et al., 2005; Feusner et al., 2009) when associated with conditions of chronically elevated dopamine activity (Berridge et al., 2005; Taylor et al., 2010), even related to DAT dysfunctions (Sanna et al., 2020). On the other hand, medium to high doses of psychostimulants such as cocaine or amphetamines totally suppress spontaneous grooming behavior in rats (Antoniu et al., 1998). Hence, we assessed the frequency of grooming behavior after acute and chronic treatment with CE-158 to assess potential effects of our treatment on this behavioral response. As shown in Figure 7, no significant differences were observed in the number of grooming episodes between rats treated with CE-158 1 mg/kg or 10

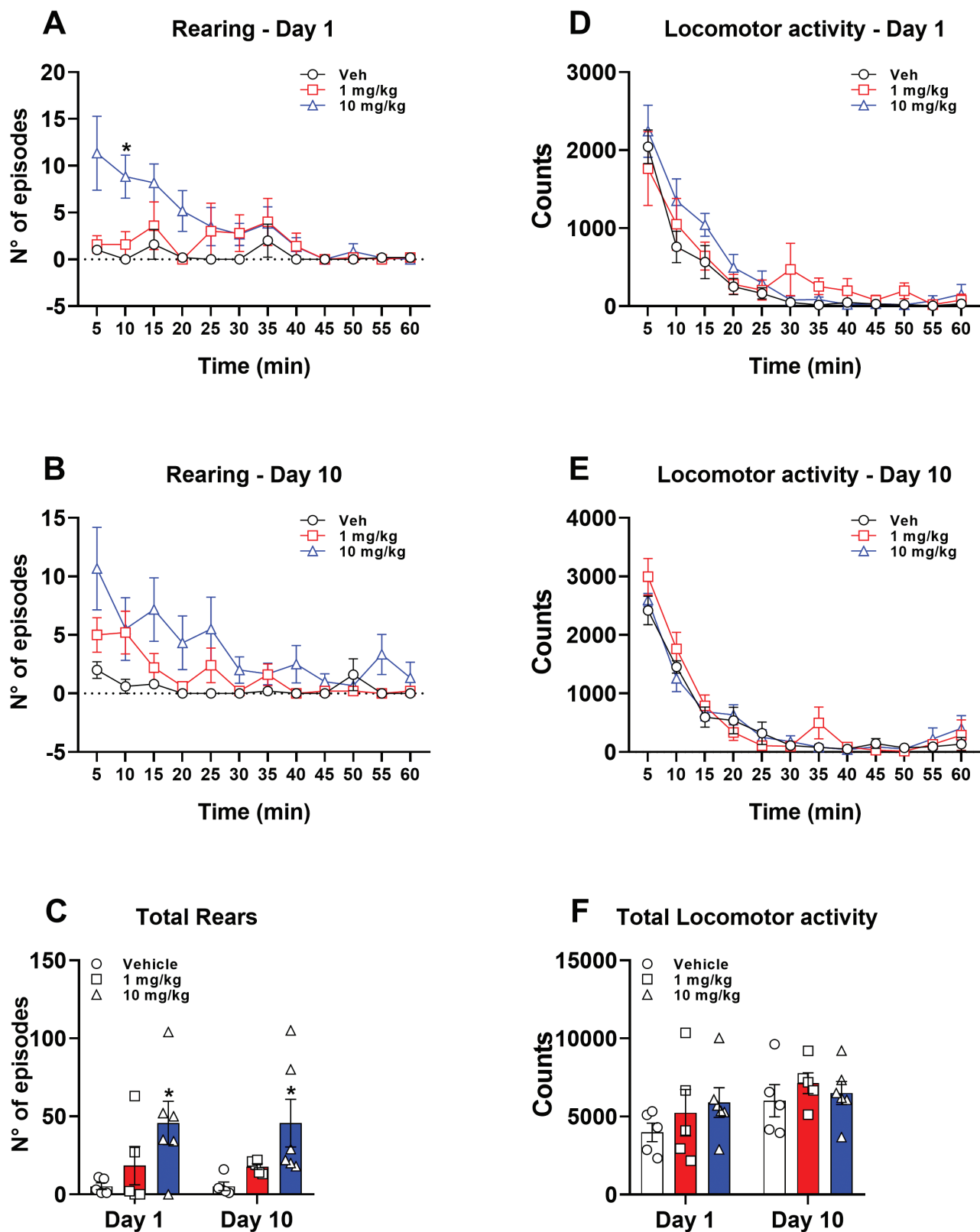


Figure 4. Rearing episodes and horizontal locomotor activity in rats treated with CE-158 1 mg/kg ($n=5$) or 10 mg/kg ($n=6$) i.p. compared with vehicle (Veh)-treated rats ($n=5$) on day 1 and day 10 of the treatment. **Figure 4A** and **4B** indicate the number of rearing episodes and **Figure 4D** and **4E** the counts for horizontal locomotor activity during the test relative to the 5 minutes (min) fractions. In **Figure 4C** and **4F** are reported the values for the entire 60 minutes test at day 1 and day 10. Values are expressed as mean \pm SEM. * $P < .05$ compared with vehicle-treated rats (RM 2-way ANOVA followed by Bonferroni multiple comparison test).

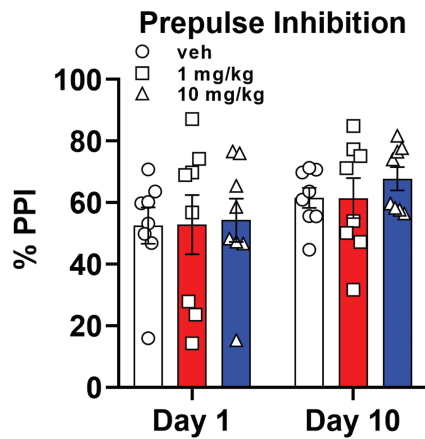


Figure 5. Assessment of prepulse inhibition (PPI) of the startle response in rats treated with CE-158 1 mg/kg or 10 mg/kg compared with vehicle (Veh)-treated rats after the first (day 1) and the last (day 10) treatment. Values are expressed in percentages (see Materials and Methods section) and are mean \pm SEM of 8 rats/group (RM 2-way ANOVA: not significant).

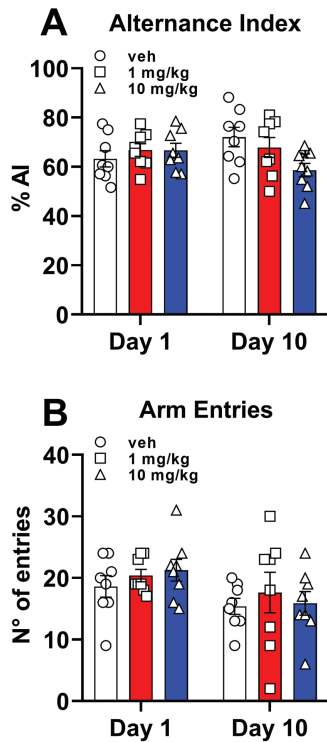


Figure 6. Alternance index (A) and the number of arm entries (B) in the Y maze in rats treated with CE-158 1 mg/kg or 10 mg/kg compared with vehicle (Veh)-treated rats after the first (day 1) and the last (day 10) treatment. Values are expressed as mean \pm SEM of 8 rats/group. * $P < .05$ compared with vehicle-treated rats (RM 2-way ANOVA followed by Bonferroni's multiple comparison test).

mg/kg and vehicle-treated rats (2-way ANOVA, $F_{(2,13)} = 0.881$, $P = .437$) nor after acute or chronic treatment (2-way ANOVA, $F_{(1,13)} = 0.312$, $P = .586$).

DISCUSSION

Here, we show for the first time, to our knowledge, that systemic administration of the new modafinil analog CE-158 significantly

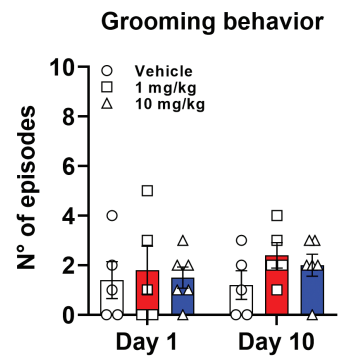


Figure 7. Grooming behavior in rats treated with CE-158 1 mg/kg ($n = 5$) or 10 mg/kg ($n = 6$) compared with vehicle-treated rats ($n = 5$) after the first (day 1) and the last (day 10) treatment. Values are expressed as mean \pm SEM (RM 2-way ANOVA: not significant).

increases dopamine neurotransmission in the mPFC of male rats. The effect is dose dependent, with a peak at around 30 minutes after treatment (about 30% and 75% above basal values for the doses of 1 and 10 mg/kg, respectively) and lasts for at least 120 minutes. The extent and time course of increased extracellular dopamine levels is similar to that observed with other DAT inhibitors like modafinil (Rowley et al., 2014), CE-123 (Sagheddu et al., 2020), MK-26 (Kouhnavardi et al., 2022), GBR12909 (Weikop et al., 2007), and methylphenidate (Berridge et al., 2006), although with the latter a greater effect was observed at comparable doses (Bymaster et al., 2002; Marsteller et al., 2002; Rowley et al., 2014). These results are also confirmed by electrophysiology experiments, which are consistent with an increased dopamine activity following drug treatment. In particular, the functional sampling showed that the low dose of CE-158 in a 10 day-chronic regimen is sufficient to activate pyramidal neurons in the mPFC. This is in agreement with our previous experiments following acute treatment with the same dose in both young and aged rats (Lubec et al., 2021) and with an acute injection of methylphenidate either i.v. or locally injected in the PFC (Gronier, 2011). On the contrary, another selective dopamine uptake inhibitor, GBR12909, was not found to activate pyramidal neurons in the mPFC when administered i.v. up to 6 mg/kg (Gronier, 2011), possibly due to a different binding mechanism to the transporter (Schmitt and Reith, 2011).

The increased average firing frequency, resulting from the prolonged CE-158 low-dose treatment, suggests an additional long-term influence on the mPFC, which could be related to the behavioral output (see below). In this brain region, dopamine exerts a modulatory action by acting at different temporal and spatial scale through the interaction with D1-like and D2-like receptors, directly modulating glutamatergic synapses at pyramidal neurons and indirectly on the activity of inhibitory interneurons (Lohani et al., 2019). An optimal dopaminergic tone influences processes such as working memory, attention, and behavioral flexibility (Ott and Nieder, 2019). The drug possibly impacts pyramidal cells, being GABA interneurons not biased by different doses. Interneurons are key within local cortical networks and specifically in the mPFC, where they coordinate several cognitive tasks, ranging from learning and memory (Kupferschmidt et al., 2022) to attention guidance (Kim et al., 2016), and related behaviors (Kvitsiani et al., 2013). Because psychostimulants have been associated with mPFC micro-circuit maladaptation in several physiopathological conditions (Lapish et al., 2015; Bisagno et al., 2016; Kuiper et al., 2017), the interneuron steadiness despite chronic treatment with CE-158 is relevant concerning the drug security profile.

The i.v. dose-response curve at the end of the 10-day treatment showed an increased neuronal firing frequency in the mPFC of pretreated rats, irrespective of the low or high CE-158 dose for pretreatment, as compared with the maximal firing induced in vehicle-pretreated rats. This further indicates the effectiveness of the chronic vs the acute regimen to sustain the activation of the mPFC.

These functional results are also corroborated by the behavioral findings indicating the ability of the compound to elicit exploratory behavior (i.e., rearing) to a similar extent after both acute and chronic treatment without any sign of tolerance to its pro-active effects, as well as its inability to induce any behavioral alterations typical of chronic psychostimulants (i.e., amphetamines and cocaine). Exploratory behavior and rearing, in particular, can be seen as a reliable discriminant for assessing the presence of cognitive enhancement by a given compound (i.e., increase in wakefulness, vigilance, and attention) without signs of excessive dopamine activity (Wood et al., 2014; Minassian et al., 2016). It is well known that sustained dopaminergic stimulation occurring with psychostimulants can lead to a profound imbalance in the functioning of the mesocorticolimbic circuit, in particular, an excessive activity of the mesolimbic pathway together with hypoactivity of the mesocortical one, with a significant decrease of dopamine-related function in the PFC (Jentsch et al., 2000; Kalivas, 2009; Goto et al., 2010; Hui and Beier, 2022). In preclinical models, alterations in mesocorticolimbic circuitry are reflected by behavioral signs such as changes in locomotor activity (i.e., behavioral sensitization), alterations in sensorimotor gating, impairments in exploratory behavior and working memory, and loss of behavioral flexibility, which reflect the presence of cognitive deficits and/or addiction-like states (Wood et al., 2014).

Our results indicate that neither single nor repeated administration of CE-158 at 1 or 10 mg/kg induced significant increase in locomotor activity. More importantly, our chronic treatment of 10 days did not induce behavioral sensitization. Behavioral sensitization is considered a typical sign of the functional alterations induced by chronic psychostimulants at the level of the mesocorticolimbic circuit. It reflects, in particular, a maladaptive imbalance between the activity of pyramidal glutamatergic neurons and other neural populations in the PFC, dopamine projections from the VTA and GABA interneurons, and it is believed to underlie the chronicization of the addictive state (Steketee, 2003).

A similar lack of effects was also observed in the PPI test evaluating sensorimotor gating. Sensorimotor gating provides a precognitive attentional filter that prevents sensory overload to ensure the proper processing of relevant sensory information, allowing correct cognitive functioning and behavioral responses to environmental stimuli (Braff and Geyer, 1990). This function is sensitive to several psychotropic drugs, particularly dopaminomimetics (Cáceda et al., 2012). One of the brain areas thought to play a key role in sensorimotor gating is the PFC (Swerdlow et al., 2001). Accordingly, PFC dysfunctions, such as those observed in schizophrenia or drug addiction, can lead to impairments of the PPI (Hazlett and Buchsbaum, 2001; Day-Wilson et al., 2006). Our results indicate that CE-158 is ineffective in altering or disrupting sensorimotor gating after acute or prolonged administration.

Spontaneous alternance in the Y maze is considered a general index of exploration, behavioral flexibility, and working memory (Kraeuter et al., 2019), and the PFC is involved in all these functions (Lalonde, 2002). Psychostimulants such as amphetamines affect spontaneous behavioral alternance in the Y maze both in mice (Cherng et al., 2007) and rats (Seyedhosseini Tamijani

et al., 2018), increasing perseverative and stereotyped behavior and impairing working memory. Similar alterations can also be observed in DAT knockout rats (Leo et al., 2018; Sanna et al., 2020), a transgenic model of chronic hyperdopaminergia. Here, we did not observe any side effect of the acute or chronic treatment with CE-158 on exploration, working memory, or behavioral flexibility as assessed through the Y-maze test. Accordingly, no difference has been detected between vehicle-treated and CE-158-treated rats in the behavioral alternance index nor in the number of arm entries. A slight, though not significant, increase in the number of arm entries was observed following the acute administration of the compound at the high dose. This result parallels the slight but nonsignificant increase in locomotor activity observed after the acute treatment, possibly reflecting a stimulatory effect on exploration, as supported by the data obtained on the rearing behavior. Likewise, these results are in agreement with the findings that CE-158 enhanced behavioral flexibility and recovered rats from scopolamine-induced impairments in behavioral flexibility as assessed by the attentional set shifting task (Lubec et al., 2021).

Finally, as excessive grooming is a reliable sign of dopamine-induced stereotyped/compulsive behavior (Hollingsworth and Mueller, 1988; Ukai et al., 1992; Antoniou et al., 1998; Berridge et al., 2005; Feusner et al., 2009; Taylor et al., 2010), even directly related to DAT dysfunctions (Sanna et al., 2020), the lack of any significant effect in grooming behavior by CE-158 suggests that at the conditions used in this study the drug is devoid of compulsive-like effects and confirms its inability to induce stereotyped behavior.

The present research has some limits. Firstly, functional changes on density and distribution of dopamine and/or glutamic acid receptors in the mPFC, have not been yet investigated, though this information can be useful in shedding light on the molecular effects of acute and chronic CE-158 (Gonzales et al., 2019). Secondly, our study has been conducted only in males, although a broad literature points out the key role of sex/gender differences in chronic use of psychostimulants both at recreational and clinical settings (Dafny and Yang, 2006; Daiwile et al., 2022; Pisanu et al., 2022). Finally, a differential impact of the anesthetics used for the microdialysis and electrophysiology experiments (isoflurane vs chloral hydrate) cannot be completely ruled out. Further studies are needed to examine the translational potential and safety profile of CE-158.

Nonetheless, our data show that CE-158 dose-dependently elicits responsiveness to environmental probing both after acute and repeated administration without inducing behavioral sensitization in consequence of chronic treatment or compulsive-like behaviors. Moreover, at the doses used, we did not detect any behavioral correlates of psychotic-like signs, such as alterations in sensorimotor gating or in behavioral flexibility and working memory, which have been repeatedly attributed to other psychostimulants (Cherland and Fitzpatrick, 1999). Accordingly, the electrophysiological and neurochemical results support the view that this compound potentiates prefrontal activity without any sign that could resemble the modifications induced by chronic psychostimulants (Bisagno et al., 2016). In conclusion, the novel atypical and selective DAT inhibitor CE-158 can be considered as a promising pro-cognitive compound and contributes to assessing its preclinical safety profile also under repeated administration for further translational/clinical testing.

Supplementary Materials

Supplementary data are available at *International Journal of Neuropsychopharmacology (IJNPPY)* online.

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None.

Interests Statement

None.

Author Contributions

C. S., F. S., J. L., P. K.: Conceptualization, Supervision, Methodology, Data curation, Formal analysis, Writing—original draft; F. S., E. C., F. B.: Investigation (microdialysis and behavioral experiments); C. S., A. L. M., M. P.: Investigation (electrophysiology); C. S., F. S., M. P., J. L., G. L.: Writing—review and editing, Validation.

Data Availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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