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Optimizing cognitive enhancement through dopamine transporter modulation

Marco Niello [a,b,c,*,1], Predrag Kalaba [d,1], Anita Cybulska-Klosowicz [e], Michael Kirchhofer [d], Iva Spreitzer [d], Markus Spreitzer [d], Judith Wackerlig-Damle [d], Michele Santoni [f,j,k], Claudia Sagheddu [j], Simone B. Sartori [g], Karl Ebner [g], Jana Lubec [f], Ahmed M. Hussein [f,m], Tamara Stojanovic [f], Nicolas Singewald [g], Marco Pistis [j,k,l], Gert Lubec [f,2], Harald H. Sitte [a,h,i,**,2]

[a] Center for Physiology and Pharmacology, Institute of Pharmacology, Medical University of Vienna, Vienna, 1090, Austria
[b] Genetics of Cognition Laboratory, Neuroscience Area, Istituto Italiano di Tecnologia, via Morego, 30, Genova, 16163, Italy
[c] IRCCS Azienda Ospedaliera Metropolitana (AOM) di Genova, Largo R. Benzi, 10, Genova, 16132, Italy
[d] Department of Pharmaceutical Sciences, Faculty of Life Sciences, University of Vienna, Vienna, 1090, Austria
[e] Laboratory of Emotions Neurobiology, Nencki Institute of Experimental Biology, Polish Academy of Sciences, Warsaw, 02093, Poland
[f] Department of Neuroproteomics, Paracelsus Medical University, Salzburg, 5020, Austria
[g] Department of Pharmacology and Toxicology, Institute of Pharmacy and Center for Molecular Biosciences Innsbruck, University of Innsbruck, Innsbruck, 6020, Austria
[h] Hourani Center for Applied Scientific Research, Al-Ahliyya Amman University, Amman, 19328, Jordan
[i] Center for Addiction Research and Science, Medical University of Vienna, Vienna, 1090, Austria
[j] Department of Biomedical Sciences, University of Cagliari, Monserrato, 09042, Italy
[k] Unit of Clinical Pharmacology, University Hospital, Cagliari, Italy
[l] Neuroscience Institute, National Research Council of Italy, Section of Cagliari, Italy
[m] Division of Pharmacology and Toxicology, Department of Pharmaceutical Sciences, University of Vienna, Vienna, 1090, Austria

A B S T R A C T

Psychostimulants act on monoamine systems including the dopamine transporter (DAT), a key regulator of dopamine reuptake following synaptic release. While these compounds can enhance energy, cognition, and sociability, their clinical utility is often limited by their abuse potential and peripheral side effects. Research has traditionally focused more on their addictive properties rather than their therapeutic potential. However, certain psychostimulants, such as R-modafinil, enhance cognitive performance without inducing significant euphoria or addiction, making them promising lead candidates for clinical application. In this study, we hypothesized that R-modafinil analogs with extended residence time at DAT (i.e., slow k_{off}) would enhance cognitive function more effectively. To test this, we evaluated a series of R-modafinil analogs using *in vitro* equilibrium and non-equilibrium measurements, *in vivo* fast-scan cyclic voltammetry, and highly translational cognitive assays in both healthy and scopolamine-treated rats modelling cognitive impairment. Our findings show that prolonging DAT occupancy improves dopamine signalling and leads to more robust enhancements in cognitive flexibility. Compounds with longer DAT resident time—such as S-MK-26 and (S,S)-CE-158—produced the strongest cognitive effects. These results highlight the importance of DAT binding kinetics in shaping the behavioural actions of psychostimulants and support the development of safer and more effective dopamine-based cognitive enhancers.

1. Introduction

The dopamine transporter (DAT) is a key protein in spatially and temporally shaping the dopaminergic signal (Cragg and Rice, 2004; Sulzer et al., 2016). As a member of solute carrier family 6 (Slc6) family, DAT reuptakes the dopamine (DA) released by exocytosis from the extracellular space and moves it back into the presynaptic terminals (Kristensen et al., 2011). The importance of DAT in the regulation of brain dopaminergic signaling is well established (Giros et al., 1996; Leo et al., 2018). Moreover, single-point mutations altering DAT function are associated with a variety of psychiatric disorders, highlighting the importance of DAT in normal and pathological conditions (Bhat et al.,

* Corresponding author. Center for Physiology and Pharmacology, Institute of Pharmacology, Medical University of Vienna, 1090 Vienna, Austria.
** Corresponding author. Center for Physiology and Pharmacology, Institute of Pharmacology, Medical University of Vienna, 1090 Vienna, Austria.
E-mail addresses: marco.niello1991@gmail.com (M. Niello), harald.sitte@meduniwien.ac.at (H.H. Sitte).
[1] equally contributing authors.
[2] equally contributing senior authors.

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2021; Hansen et al., 2014; Herborg et al., 2021; Mazei-Robison et al., 2008). DAT is also the target of important pharmacological agents, classified as inhibitors if they bind to the transporter and prevent neurotransmitter uptake, or as releasers if they act as substrates and reverse the normal direction of transporter flux (Reith and Gnegy, 2019; Sitte and Freissmuth, 2015). Regardless of their mechanism of action as inhibitors or releasers, many compounds interacting with DAT stimulate different brain functions and are normally classified as psychostimulants (Rasmussen, 2015; Sitte and Freissmuth, 2015). Psychostimulants produce variable effects depending on the dose and the substance administered. In general, low doses produce positive effects such as increased energy, cognitive enhancement, locomotion, sociability, while reducing fatigue and appetite. Moderate doses are instead associated with cognitive impairment and euphoria, while high doses may lead to severe side effects including cardiovascular and hematologic problems (Boutrel and Koob, 2004; Gopinath et al., 2023; McCreary et al., 2015; Miller and Khoshbouei, 2024; Rasmussen, 2015; Wood et al., 2013). Drugs that act more potently with DAT compared to the serotonin transporter (SERT) are typically associated with higher psychostimulant and abuse potential (Ritz et al., 1987; Wee et al., 2005). Therefore, the DAT/SERT selectivity ratio is regularly used to predict the abuse liability of transporter ligands (Bauer et al., 2013; Luethi and Liechti, 2020). However, some DAT inhibitors, such as cocaine, despite showing an unselective DAT/SERT profile, still show pronounced psychostimulant effects and high abuse liability when used in pharmacodynamically active dosage and appropriate administration route. On the other hand, benztropine possesses high DAT selectivity but lacks pronounced reinforcing properties. This absence of reinforcement has been attributed to its atypical binding mode: benztropines stabilize the inward-facing conformation of DAT, which differs from the typical binding mode of classical DAT inhibitors such as cocaine (Chen et al., 2004; Li et al., 2024; Newman et al., 2021). The case of cocaine and benztropine suggests that while the DAT/SERT ratio is still an important tool for general toxicological predictions, it does not always hold true, and it highlights a fundamental gap in our understanding of psychostimulants mechanism of action (Reith et al., 2015). The present evidence points out that, despite the multitude of effects elicited by psychostimulants, basic research has mainly focused on modelling abuse-dependence (de Wit and Stewart, 1981; Koob et al., 1997; McCreary et al., 2015), drastically limiting the potential medical use of these drugs. As a matter of fact, several psychostimulants have been used for both medical and recreational purposes. Some of these drugs, such as modafinil or (S)-amphetamine, promote wakefulness without producing marked euphoria, therefore finding use in the treatment of different types of sleep disorders, including narcolepsy and obstructive sleep apnea (Mereu et al., 2013; Plazzi et al., 2024). Interestingly, modafinil recently gained popularity as a "cognitive enhancer" among healthy individuals due to its ability to improve attention and productivity (Müller et al., 2013). However, the exact mechanism underlying modafinil's effect on sleep and attention is still not fully understood. Modafinil is generally recognized as a weak atypical DAT inhibitor (Loland et al., 2012). Still, the weak interaction with DAT and the existence of related analogs with even lower DAT affinity and more powerful eugeroics properties made questioning the importance of DAT in the cognitive enhancing properties elicited by eugeroics (Mignot et al., 1994; Wisor, 2013). Recently, a new modafinil analog (S,S)-CE-158 was shown to lack off-target effects over a wide range of clinically relevant targets, and to exert promnestic effects, including reversal of cognitive decline in aged rats (Lubec et al., 2021) and improvement of social memory in mice (Ebner et al., 2022), positioning DAT as the central and clinically relevant target responsible for the cognitive effects elicited by these drugs.

Accumulating evidences are showing that the faster is the DA increase induced by a given psychostimulant, the stronger is the high perceived, and therefore the risk of misuse (Abreu et al., 2001; Resnick et al., 1977; Tomasi et al., 2023). On the other hand, the lower is the k_{off} of psychostimulants at DAT (i.e. the longer the psychostimulant stay bound to DAT) the longer is the behavioral effect (Niello et al., 2023). All together, these data suggest that while the rate at which psychostimulants act on DAT influences the qualitative effect of psychostimulants, the rate at which they unbind at DAT influences the persistence of their pharmacological effect.

Here, we applied a recently developed electrophysiological protocol (Niello et al., 2023) for quantifying the k_{off} of different modafinil analogs designed for cognitive enhancement and determined whether stabilizing the DAT-inhibitor complex could lead to improved cognitive enhancement.

We show that more effective cognitive enhancers display a higher affinity for DAT and that the increased affinity is associated with a slower k_{off}. *In vivo* fast scan cyclic voltammetry (FSCV) experiments revealed that the high affinity DAT inhibitors impact DA dynamics in anhestetized rats. Moreover, in agreement with the resident-time model (Copeland, 2016; Copeland et al., 2006), the compounds with a slower k_{off} also had a higher impact on cognitive flexibility in rats, highlighting the applicability of our approach for the development of DAT inhibitors with therapeutic application.

2. Experimental procedures

2.1. Animals

The experiments were carried out on 40 adult male Wistar rats, weighting 200-250 g at the arrival. The animals were group-housed under standard humidity and temperature conditions on a 12 h light/dark cycle. Behavioral testing was performed during the light. Until the testing in the ASST rats were given free access to water and pellet food. All of the procedures were in accordance with the European Community Council Directive (2010/63/EU) and approved by the 1st Local Ethics Committee for Animal Experimentation in Warsaw (ethics permits no. 1085/2020, 1237/2021 and 1526/2023).

2.2. Cell culture

Cells were kept in culture as described previously (Niello et al., 2021). In brief, human embryonic kidney 293 cells (HEK293 cells) were cultivated in Dulbecco's Modified Eagle Medium (DMEM), containing 10% fetal calf serum (FCS). Stable cell lines were generated by transfecting HEK293 cells with DAT/SERT/NET plasmid with polyethylenimine (Santa Cruz) at a ratio of 1:3 (w/w) in serum-free DMEM, and selected using Geneticin. Cells were then maintained in a sub-confluent state (37 °C, 5% CO2). For uptake or uptake-inhibition experiments cells were seeded the day before the experiment onto poly-D-lysine (PDL) coated 96-well plates. In the case of transporter-electrophysiology cells were instead seeded at low density onto PDL-coated 3 cm dishes.

2.3. Drugs

For *in vitro* experiments all the compounds were dissolved in water with 10% DMSO, and then diluted at least 1:10 in appropriate buffer for preparing working solutions. For *in vivo* experiments instead they were prepared in Kolliphor EL 30% at a nominal concentration of 1 mg/mL. The formulation was then stirred for 5 min at 90 °C, then sonicated for 5 min. This stirring/sonication cycle was repeated twice. The obtained formulation was then stirred for 1 min at 90 °C and left under stirring until administration. All the formulations were prepared and used the same day of the experiments.

2.4. Radiotracer assay

Experiments were conducted as described previously (Niello et al., 2021).

In brief, on the day of the experiment, cell culture medium was

replaced with Krebs-HEPES buffer. In the case of saturation of uptake experiment, cells were pre-incubated with sub-saturating concentrations of the inhibitor. After 10min the cells were exposed for 1min or 6min with tritiated substrates (0.2 μM [^{3}H]DA for DAT and 0.2 μM [^{3}H]5-HT for SERT), in presence of the inhibitor, then washed with ice-cold KHB and lysed with 1% sodium dodecyl sulphate (SDS). The radioactivity was determined with a beta-scintillation counter. Non-specific uptake was determined in the presence of 30 μM methylphenidate (DAT-inhibitor) or 10 μM paroxetine (SERT-inhibitor) and subtracted. For uptake-inhibition experiments, the uptake in the absence of the substance of interest was defined as 100% uptake. IC50s were determined by non-linear regression fits (GraphPad Prism 10.0).

2.5. Electrophysiology

Transporter-mediated currents were recorded in whole-cell configuration using HEK293 cell line stably expressing DAT previously developed (Sitte et al., 1998). Cells were clamped at −60mV and continuously superfused with a physiological external solution (140 mM NaCl, 2.5 mM CaCl2, 2 mM $MgCl_2$, 20 mM glucose and 10 mM HEPES, pH = 7.4). The pipette solution contained 133 mM potassium gluconate, 6 mM NaCl, 1 mM CaCl2, 0.7 mM MgCl2, 10 mM HEPES, 10 mM EGTA, pH = 7.2. Currents elicited by DA, were measured at room temperature (20-24 °C) using an Axopatch 700B amplifier and pClamp 11.2 software (MDS Analytical Technologies). All the solutions perfused onto the cell were applied using a DAD-12 superfusion system and a 8-tube perfusion manifold (ALA Scientific Instruments). Current traces were filtered at 1 kHz and digitized at 10 kHz using a Digidata 1550 (MDS Analytical Technologies). Current amplitudes in response to substrate application were measured using Clampfit 10.2 software (Molecular Devices). For the analysis, passive holding currents were subtracted, and the traces were filtered using a 100-Hz digital Gaussian low-pass filter.

2.6. Compound isolation form brain and blood and pharmacokinetic analysis

Blood and brain analysis of the compounds were performed as described previosly (Lubec et al., 2021). The pharmacokinetic study of S-CE-123 and (S,S)-CE-158 was outsourced to Evotec France (Study code DMPK_2019_045 and DMPK_2018_049), while the study on R-modafinil and S-MK-26 was performed in-house. In brief, male Sprague Dawley rats (Janvier, 314-364 g) were group-housed in dedicated home cages (1291H; size 820 cm2, Tecniplast ®) and maintained in controlled environment (22 ± 2 °C) with 12h/12h light dark cycle. Rats were anesthetized with isofluorane, and implanted with a catheter in the vena cava. Rats were pretreated by gavage with the different compounds at 10 mg/kg, using a plastic syringe fitted with a stainless steel oral cannula. Samples were taken at 15, 30 min, 1, 3, 7, 10, and 24 h either with a jugular vein cannula for blood plasma (serial) or at end point for blood plasma and total brain lysates (destructive). Brain were weighted and ground using Precellys 24 or Precellys Dual equipment (Bertin Technologies, France). For each brain correctly weighed, 4 volumes of water were added to ensure an efficient grinding (diluted 5-fold post-grinding). Compounds were subsequently quantified by liquid chromatography–tandem mass spectrometry (LC–MS/MS, Accela™ UHPLC system coupled Thermo TSQ quantum Altis and Excalibur V.3.0 software) and liquid chromatography-high-resolution mass spectrometry (LC-HRMS, Dionex UltiMate 3000 RSLC-series system, Thermo Fisher Scientific, Inc., Germany, coupled to maXis HD Qq-TOF, Bruker Daltonics, Germany). The details on bioanalysis conditions of rat plasma and brain samples are presented in Supplementary Material. PK parameters were calculated from the plasma concentrations using the program WinNonLin 7.0 (Phoenix 64) and PKanalix 2024R1 (Lixoft SAS, a Simulations Plus company). A non-compartmental analysis with extravascular administration was performed. The linear trapezoidal method was applied for integration. Since PK parameters were calculated per individual animal, and only then averaged across subjects, refer to the Supp. Table 2.

2.7. In vivo fast scan cyclic voltammetry (FSCV)

All of the procedures were in accordance with the European Community Council Directive (2010/63/EU) and approved by the Italian Ministry of Health (n° 953/2024-PR). Sprague Dawley rats were anesthetized by intraperitoneal injection of urethane (1.3 g/kg; Sigma-Aldrich, USA). Rats were placed in a stereotaxic apparatus (Kopf, Tujunga, CA, United States). Openings were made in the skull and a carbon fiber working electrode (32 μm diameter, 300 μm exposed length) was inserted into the dorsal striatum caudate/putamen (AP:1.6 mm, ML: 2.0 mm, DV: −5.0 mm). A bipolar stimulating electrode (diameter 0.35 mm) was inserted into the medial forebrain bundle (MFB; AP:-2.2 mm, ML: 2.0 mm, DV: −7.5 to −8.8 mm). The depth of the stimulating electrode was adjusted to evoke maximal DA release. An Ag/AgCl reference electrode was placed on the skull via a saline bridge. The MFB was stimulated by applying 1s, 200 μA, 60Hz, and 2 ms in duration, which produced a detectable movement of the whiskers. The applied potential of a triangular waveform was ramped up from −0.4 to 1.2 V and back to −0.4 V at a scan rate of 300 V/s vs. an Ag/AgCl reference electrode. Waveform generation and data collection were performed with the Invilog Voltammetric System and Software (Acquisition and Stimulation A&S, Invilog Research Ltd, Kuopio, Finland) and analyzed by a Fast Cyclic Voltammetry Analysis (FSV Analysis, Invilog Research Ltd, Kuopio, Finland). We used 5–6 rats per group. During the experiment, a stimulus of 1s, 200 μA, 60Hz, and 2 ms in duration, was applied each 5 min throughout the entire period of recording, which was characterized by at least 15 min period of vehicle, and by 120 min after compound administration. In the present study, DA was used as the standard to calibrate the carbon fiber working electrode sensitivity. Therefore, the peak oxidation currents for DA in each voltammogram (at approximately 0.65 V) were converted into concentration from a post-experiment calibration against fresh solutions of 1, 2, 3uM DA.

2.8. Attentional set-shifting task (ASST)

Rats were handled for two weeks prior to the beginning of drug administration. Data for (S,S)-CE-158, including the (S,S)-CE-158 + scopolamine condition, were obtained from a previously published cohort (Lubec et al., 2021). For the present study, the same experimental protocol was used as in the original work—including compound preparation, vehicle composition, administration route, and testing procedures—to ensure full methodological consistency across cohorts. Vehicle and the three treatments S-CE-123, S-MK-26 and R-Modafinil (1 mg/kg) were administered via oral gavage in four separate groups every day for one month and then 30min prior to testing in each of ASST stages. After 2 weeks of washout period, animals were tested again in the ASST, following the administration of the amnestic antimuscarinic drug Scopolamine (1 mg/kg, i.p., 30min prior testing). The test apparatus was a Plexiglas box (38 × 58 × 31 cm), with two “choice” sections, where glass digging pots were placed. The glass pots were filled with media of different textures (plastic pellets, wool, sawdust, gravel, glass marbles, coarse sand, straws, paper strips, thick threads and synthetic raffia) and different scents (lemon, almond, orange, cream, vanilla, chocolate, arrack, green tea, lavender, rum). During testing one of the bowls was baited with a small piece of honey breakfast cereal (Vitanella, Poland) buried under the digging media. Rats were required to determine which of the two bowls was baited using either the texture or the odor of the medium as cues and learn to respond to the relevant cue. Before testing rats were habituated to the bowl filled with the bait (reward) in their home cages for 24 h and to the testing apparatus and glass bowls for 10 min per day for 3 days. Unscented non-discriminable digging media were used for the acclimatization period. After habituation animals were moved to the first stage of the ASST. The animals had no access to food

for ~12 h before testing. Water was available ad libitum. All rats were trained on the same discriminations and in the same order during testing. The order of the training discriminations, the reinforced odor and medium and their left or right position in the test apparatus were determined pseudo-randomly in a counterbalanced manner. Digging was defined as active digging with both front paws or active foraging with the snout in the digging medium. Rats were allowed 3 min of exploration; if the response (digging) did not occur in that time the trial was terminated. If the rat dug in the non-rewarded bowl an error was recorded and the trial was terminated.

In the simple discrimination stage (SD) the bowls differed along one of two dimensions (e.g., odor), rats were trained in discrimination of two different odors and had to learn to associate the food reward with a specific odor. The complex discrimination stage (CD) had the same correct (reinforced) and incorrect (non-reinforced) stimulus properties as the SD, but a new irrelevant dimension (i.e., medium texture, or "medium" in short) was introduced. For the reversal stage (Rev), the relevant dimension and the stimuli were unchanged, however the previously correct stimulus was now non reinforced. The intra-dimensional shift phase (IDS) required a shift of attention within the dimension. In this stage both correct and incorrect stimuli changed, but the relevant dimension remained the same (e.g. odor dimension is still rewarded vs medium dimension, hower the odors that are rewarded are changing, as well as the media. Fig. 4a, "IDS"). In the extra-dimensional shift phase (EDS) shifting of attention between dimensions was required. In this stage the correct and incorrect stimuli changed again, but the originally relevant dimension became irrelevant and the formerly irrelevant dimension became relevant. So, if odor had previously been a reliable predictor of the location of the reward, now the texture of the digging medium would become the relevant dimension (e.g., odor and media are both changing again, and the media discrimination become rewarded instead of the odor discrimination Fig. 4a, "EDS".).

During the second testing in the ASST (after 2-weeks of drug washout) new digging media and odors were used.

Rats performed each of the ASST test stages in a single test session (single day) or the ASST test stage procedure was divided into two sessions (two consecutive days). The two-session procedure was introduced when needed in the difficult stages of the test (Rev, EDS), where animals needed many more trials to reach the criterion. Stages CD, IDS, Rev and EDS started with few (~3) trials from the previous stage to be sure that rats remember the cue from the previous day.

Behavior was monitored by a camera and stored to be analyzed offline if needed.

For each test stage, the number of trials required to achieve to criterion of six consecutive correct responses was computed.

2.9. Statistics

Comparisons between two independent groups were performed using two-tailed unpaired t-tests ($\alpha = 0.05$). For analyses involving more than two groups, we used a one-way ANOVA followed by Tukey's post hoc multiple-comparison test ($\alpha = 0.05$). To evaluate treatment effects across ASST stages, we applied a two-way mixed-effects ANOVA with Šídák's correction or Uncorrected Fisher's LSD for multiple comparisons. Uncorrected Fisher's LSD was employed to identify preliminary patterns. Normality of data distributions was assessed using the Kolmogorov–Smirnov test. p values and related statistics are reported only for comparisons that reached statistical significance.

3. Results

3.1. Binding kinetics optimization of DAT inhibitors

We evaluated the ability of different modafinil analogs (Fig. 1a) to inhibit the reuptake of [^{3}H]-DA, [^{3}H]-MPP+, and [^{3}H]-5HT in HEK293 cells expressing the human DAT, norepinephrine transporter (NET) or SERT, respectively and compared them to R-modafinil, the pharmacologically active enantiomer of modafinil. Apart from cocaine, none of the compounds showed pharmacologically relevant activity at NET and SERT, indicating a strong selectivity for DAT (Fig. 1b–d; Supp. Table 1.). Instead, based on their activity at DAT, two major groups of compounds could be identified. The first group displayed DAT inhibitory potency comparable to that of R-modafinil (Fig. 1b; IC50 R-modafinil: 6.59 μM, 95% CI: 4.87–8.88; S-CE-123: 4.43 μM, 95% CI: 3.46–5.61; CE-125: 4.20 μM, 95% CI: 3.42–5.11; S-CE-146: 1.14 μM, 95% CI: 0.88–1.47; R-CE-146: 2.21 μM, 95% CI: 1.70–2.90). The second group was characterized by markedly higher potency, with IC50 values < 0.5 μM and therefore an approximately 30-fold leftward shift relative to R-modafinil ((S,S)-CE-158: 0.21 μM, 95% CI: 0.18–0.27; S-MK-26: 0.37 μM, 95% CI: 0.25–0.54). This latter group thus exhibited a cocaine-like affinity for DAT while remaining devoid of activity at NET and SERT (cf Fig. 1b and c–d; Supp. Table 1).

Next, we examined the dissociation rate of inhibitors from DAT using whole-cell patch clamp in HEK293 cells as performed previously (Niello et al., 2023). We elicited DAT-mediated currents by applying a saturating concentration of DA (30 μM) in hDAT-expressing HEK293 cells (Fig. 1e; the white bar ("DA 30 μM") indicates the time window during which dopamine was applied). Once established a steady-state DAT-mediated current, we co-applied the compound of interest (grey bar, indicating "+ Inhibitor", at a saturating concentration) which inhibits the current back to baseline, according to its ability to compete with DA for the DAT binding site (grey dashed box; Fig. 1e). Following the establishment of a stable current reversal, the application of the inhibitor is stopped and the application of 30 μM DA continues alone, restoring the current amplitude (right dashed box, Fig. 1e): The rate of recovery of the current is a measure of the k_{off} for the tested inhibitor. As established earlier (Niello et al., 2023), the dopamine-mediated current recovers in a concentration-independent manner since a compound's k_{off} is solely time-dependent (s^{-1}). Non-linear regression of the current recovery showed substantial differences in the k_{off} of the tested inhibitors, with (S,S)-CE-158 and S-MK-26 showing approximately 10 times slower k_{off} compared to other drugs (Fig. 1f). The differences in the k_{off} linearly correlated with the IC50s obtained in uptake-inhibition ($R^2 = 0.5829$; F8.386, $p = 0.0275$), suggesting that, indeed, the improvement in the IC50 of the drugs depends on the establishment of a more stable DAT-inhibitor complex (Fig. 1g).

The protocol used to estimate the k_{off} of the different inhibitors enables also to evaluate if the interaction between the different compounds and DAT is dependent on one or more states of the transport cycle (Bulling et al., 2012; Gradisch et al., 2024; Niello et al., 2023). Contrary to the recovery of the steady-state currents, the inhibition of DA steady state current is concentration dependent. If the compound's action at DAT depends only on one state of the transport cycle, inhibition of the DA steady-state current saturates at the rate-limiting step (approximately 2 s^{-1} – dashed box Fig. 1h), while if it depends on more states it supersedes this rate, similar to the case of the hallucinogenic drug ibogaine (Bulling et al., 2012; Gradisch et al., 2024; Niello et al., 2023). Therefore, we tested the compounds with fastest k_{off} (R-modafinil and S-CE-123) and those with the slowest k_{off} (S-MK-26 and (S,S)-CE-158) for their DAT state dependence. All compounds saturated at the rate-limiting step of DAT (Fig. 1i), indicating their molecular pharmacological properties depend on a single state of DAT transport cycle, and differ from the hallucinogen ibogaine. Despite these results, we cannot exclude the possibility that the compounds stabilize a particular transporter conformation, leading to atypical mechanisms of action (Keighron et al., 2023; Reith et al., 2015; Schmitt et al., 2013).

3.2. In vivo pharmacological manipulation of DA dynamics

Next, we evaluated the ability of DAT inhibitors (R-modafinil, S-CE-123, S-MK-26, and (S,S)-CE-158) with a more stable DAT interaction (slower k_{off}) to influence DA dynamics *in vivo*. We compared inhibitors

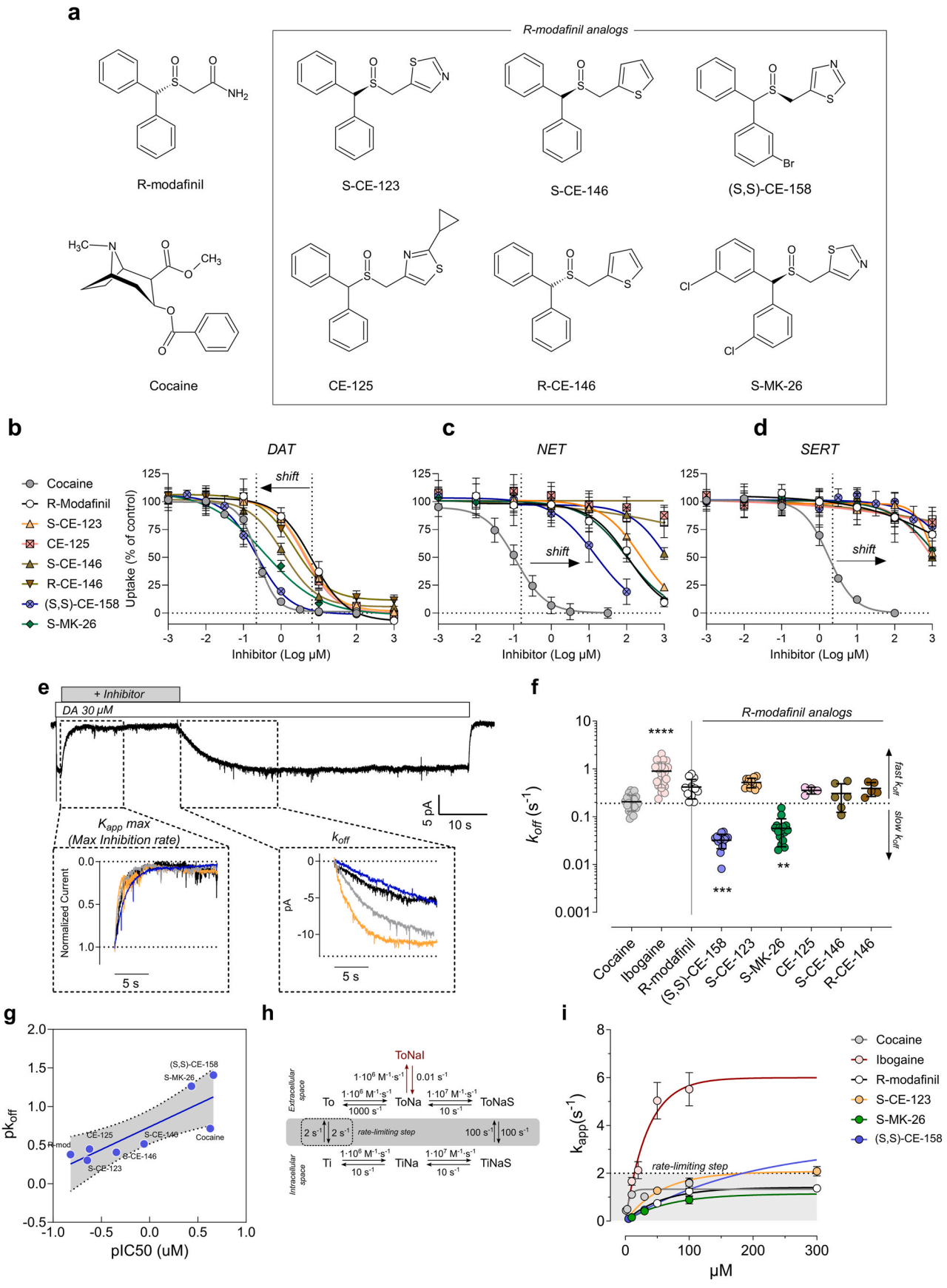

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Fig. 1. DAT inhibitors with improved pharmacodynamics.
a) chemical structures of modafinil analogs in comparison to R-modafinil and the classical psychostimulant cocaine; b) uptake-Inhibition curves in HEK293 cells expressing the human DAT, c) NET, and d) SERT; e) Representative trace(s) of whole-cell mediated currents at DAT in HEK293 cells; The two bottom insets represent example traces of current-inhibition (left) used to extrapolate Kapp, and example traces of current disinhibition (right) used to extrapolate the respective k_{off}. Example trace in black: R-modafinil; grey: cocaine; orange: S-CE-123; blue: (S,S)-CE-158; f) k_{off} from different inhibitors. Statistics is One-way ANOVA followed by Tukey multi-comparison test, $\alpha = 0.05$. ANOVA: $F(8, 104) = 21.31$, $P < 0.0001$. Tukey multi-comparison test: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$; g) Linear regression between IC50 and k_{off}, $y = 0.9774*x + 0.7533$, $R^2 = 0.5829$; F8.386, $p = 0.0275$); h) Schematics of DAT transport cycle, with estimated transport rates; i) Conformation dependence measured in whole-cell patch-clamp.

with a more stable DAT-interaction (i.e. lower k_{off} (S,S)-CE-158 and S-MK-26) to those with a less stable DAT interaction (i.e. higher k_{off} R-modafinil and S-CE-123). Using FSCV, we measured each compound's ability to inhibit the reuptake of electrically evoked DA *in vivo* in the dorsal striatum of anesthetized rats. All compounds were administered i.p. at a dose of 10 mg/kg.

As shown in Fig. 2a, electrical stimulation evoked a characteristic DA peak, identified by an oxidation peak at ~0.65V and a reduction peak at −0.4V (Figs. 2a and 3 dimension plots). Both (S,S)-CE-158 and S-MK-26 enhanced electrically evoked DA more effectively than R-modafinil and S-CE-123.In particular, while the effects of R-modafinil and S-CE-123 fade within 120 min of recording, S-MK-26, and (S,S)-CE-158 still show elevated DA_{max} at 120 min, indicating a prolonged effect (Fig. 2b–d). Notably, all compounds rapidly increased DA levels in the dorsal striatum (Fig. 2b–d). However, despite S-MK-26, and (S,S)-CE-158 displaying similar *in vitro* IC50 and k_{off} values (see Fig. 1b–f and Supp. Table 1), S-MK-26 was markedly less effective than (S,S)-CE-158 in increasing striatal DA (compare Fig. 2c and d).

3.3. Oral pharmacokinetics of DAT inhibitors

Since psychostimulants show reduced toxicity when administered orally compared with systemic parenteral administration (Bruggisser et al., 2011), we chose the oral route for the experiments. We compared the pharmacokinetics of S-CE-123, S-MK-26, and (S,S)-CE-158 with R-modafinil following their oral administration in rats before assessing their effect on cognitive performances (Fig. 3a and b). All compounds showed a slower pharmacokinetics compared to R-modafinil (cf Fig. 3a with Fig. .3b–d), a lower plasma Cmax compared to R-modafinil (R-modafinil = 1992 ± 1339 ng/mL; S-CE-123 = 669 ± 364 ng/mL; S-MK-26 = 1159 ± 509 ng/mL), and a brain:plasma ratios ≥1 which indicates good brain penetrance (Fig. 3e–Supp. Table 2). In particular, the brain:plasma ratio for S-MK-26 and (S,S)-CE-158 was even greater than 1, suggesting an active transport into the CNS. When looking at the tail of the PK curves instead, we observed results in line with our *in vitro* findings: both S-MK-26 and (S,S)-CE-158 showed a much slower excretion compared to S-CE-123 and R-modafinil. It was not possible to calculate the half-life of (S,S)-CE-158 since 5h following oral administration, the compound was still showing stable plasma and brain values (Fig. 3d).

3.4. Behavioral efficacy and suitability of slow binding kinetic DAT inhibitors

We then evaluated if analogs having a more stable interaction with DAT were also having a better therapeutic outcome. In particular, to assess the impact of the different compounds on cognitive functions, we used the ASST in Wistar rats, a behavioral task modeled after the intradimensional/extradimensional component of the Cambridge Neuropsychological Test Automated Battery (CANTAB), which is used to identify cognitive dysfunction in humans and non-human primates (Nagahara et al., 2010; Rock et al., 2014). Since (S,S)-CE-158 had been

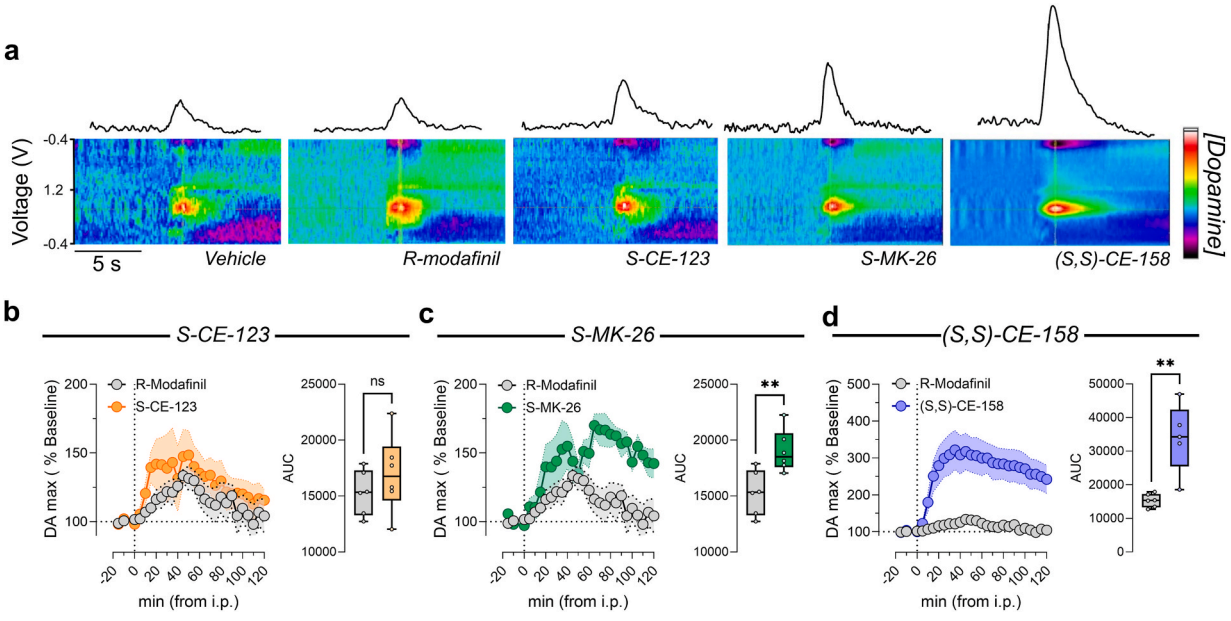

Fig. 2. in vivo pharmacological manipulation of DA dynamic.
a) Representative *in vivo* FSCV DA dynamics following the i.p. administration of the specific DAT-inhibitor; b-d) Effect over time on striatal electrically-evoked DA of the different compounds (left) and relative AUC (right) analyzed with two-tailed unpaired *t*-test, $\alpha = 0.05$ for b) S-CE-123 vs R-modafinil: $t(10) = 1.018$, $p = 0.3328$, c) S-MK-26 vs R-modafinil: $t(10) = 3.323$ $p = 0.0077$, d) (S,S)-CE-158 vs R-modafinil: $t(9) = 4.389$ $p = 0.0017$. Data are shown as mean ± SEM from 5 to 6 rats per group; d) Correlation between the max DA signal measured with *in vivo* FSCV and the *in vitro* k_{off} measured in HEK293 cells expressing DAT. Both measures are relative to R-modafinil; linear regression: $y = -0.7943*x + 1.928$, $R^2 = 0.4897$, $F = 1.920$, $p = 0.3002$; non-linear regression: y=(y0 - Plateau)*exp(-k*x) + , with y0 = 147.0, Plateau = 1.056, k = 49.95, half-life = 0.01388, $R^2 = 0.9950$.

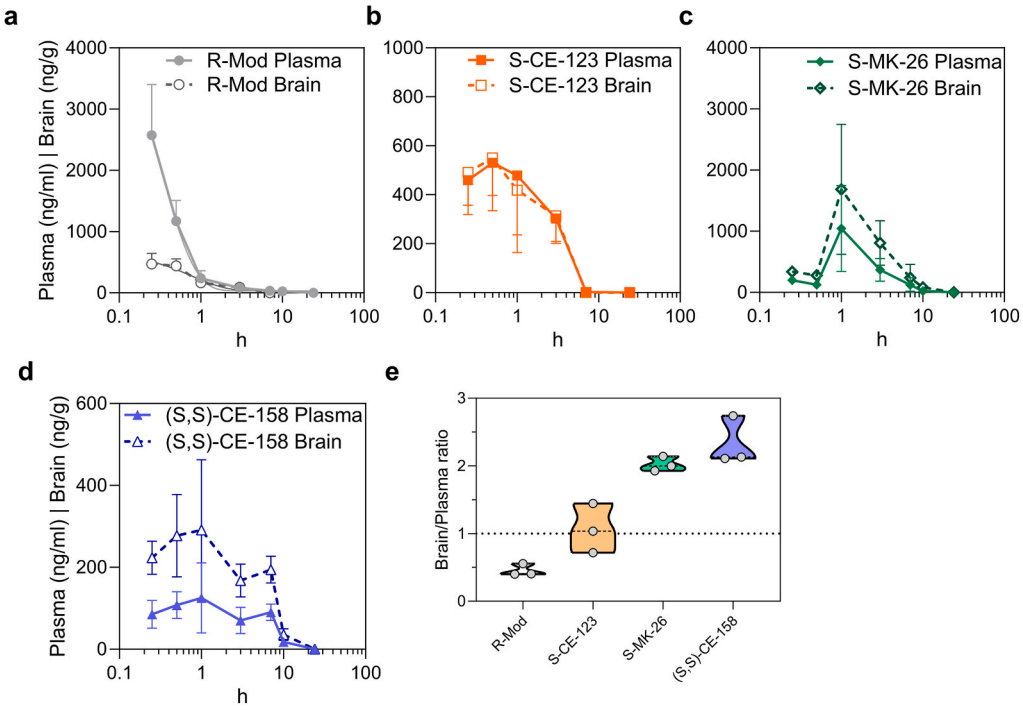

Fig. 3. PK of modafinil analogs with improved in vitro pharmacology.
a) Brain and Plasma concentration following oral administration of R-modafinil, b) S-CE-123, c) S-MK26, and d) (S,S)-CE-158; Data in a-d are mean ± SD. e) Brain/Plasma ratios of the four compounds shown as median and quartiles.

tested previously, we adhered to the 3R principles by re-using the existing dataset—including the (S,S)-CE-158 + scopolamine condition—and integrating it with the newly generated datasets for the three additional compounds (R-modafinil, S-CE-123, and S-MK-26 - see Methods). To mimic clinical conditions, compounds were given orally (gavage) once daily at 1 mg/kg for one month, and 30 min before each ASST stage (see Methods for details). In brief, rats are challenged for the discrimination of different stimuli (different odors and/or different mediums, with odors and mediums referred to as different dimensions of diverse stimuli) following the oral administration of a vehicle or a drug. The number of trials to reach the criterion (i.e. making the right choice for 6 consecutive times) is quantified as a measure of their cognitive performance. The fewer the number of attempts, the better the cognitive performance for a given ASST stage. Over time, rats were challenged with different discrimination tasks (Fig. 4a and b). In the EDS stage, shifting attention between dimensions is required (e.g., shifting attention between odor discrimination and media discrimination; Fig. 4a and b), resulting in an overall increase in the number of trials to reach the criterion vs the IDS (Fig. 4b_1). The difference in the number of trials to reach the criterion between IDS and EDS is a measure of cognitive flexibility. In particular, the smaller the difference, the greater the cognitive flexibility. (Fig. 4b_1, inset).

When tested in healthy rats, R-modafinil reduced the number of trials to reach the criterion in the EDS (Fig. 4c_1: Vehicle = 21.75, R-mod = 16.88, p = 0.0517). Despite the IDS→EDS shift cost was still present (i.e. animal still showed a higher number of trials to perform the EDS vs the IDS phase; Fig. 4c_1), R-modafinil facilitated overcoming this cognitive bias showing a statistically significant reduction in the trials to reach criterion in the EDS (Fig. 4c_2; p = 0.0424). On the contrary, S-CE-123 did not show any relevant effect compared to Vehicle in any of the cognitive dimensions tested (Fig. 4d). S-MK-26 showed statistically significant effects in the reversal (Rev; Fig. 4e_1: Vehicle = 18.00, S-MK-26 = 13.44, p = 0.0480) and in the EDS stage compared to vehicle (Fig. 4e_1: Vehicle = 21.75, S-MK-26 = 15.67, p = 0.0035). Therefore, as observed for R-modafinil, although the IDS→EDS shift cost for S-MK-26 remained evident (Fig. 4c_1: Vehicle = 10.6, S-MK-26 = 15.6, p = 0.0087), S-MK-26 nonetheless facilitated overcoming the IDS→EDS cognitive bias, as shown by a statistically significant reduction in the trials to reach criterion during the EDS (Fig. 4c_2: EDS Vehicle vs. S-MK-26 p = 0.0051). (S,S)-CE-158 did not show statistically different effects in any of the ASST phases included the EDS (Fig. 4f_1: Vehicle = 21.75, (S,S)-CE-158 = 12.33, p = 0.1270). However, despite not statistically significant vs Vehicle, (S,S)-CE-158 reduced the IDS→EDS shift cost (Fig. 4f_2: Vehicle IDS vs EDS p = 0.0002, while (S,S)-CE-158 IDS vs EDS p > 0.05). Overall, these data show a general effect of DAT inhibitors on cognitive functions, with a particular relevant effect in attenuating the consolidation of an attentional strategy.

Therefore, we assessed whether the improved DAT inhibitors could restore cognitive performance in scopolamine-treated rats, a widely used preclinical model of cognitive deficits (Barbosa et al., 2010; Flood and Cherkin, 1986; Klinkenberg and Blokland, 2010). Following two weeks of washout, we re-tested the animals after the pre-treatment with the amnestic antimuscarinic drug scopolamine (1 mg/kg i. p., Fig. 5a). Scopolamine treatment impaired the cognitive performance of rats in the IDS as evidenced by the increase in the number of trials to reach criterion (Fig. 5b and Supp.Fig. 1a).

In scopolamine-treated rats, R-modafinil showed a robust decrease in the required trials to reach criterion during the EDS (Fig. 5c_1, Vehicle = 24.3 vs R-modafinil = 15, p < 0.0001) resulting in an blunted IDS→EDS shift vs vehicle (Fig. 5c_2: EDS Vehicle vs R-modafinil p = 0.0001). S-CE-123 showed a similar result to R-modafinil with a reduction in the number of trials to reach criterion in the EDS vs Vehicle (Fig. 5d_1: Vehicle = 24.3 vs S-CE-123 = 17.7, p = 0.0033). However, despite no direct comparison was planned, it seemes that S-CE-123 has lower impact vs R-modafinil in blunting the IDS→EDS shift cost (Fig. 5d_2: EDS Vehicle vs S-CE-123, p = 0.26).

On the other hand, S-MK-26 produced a robust reduction in the number of trials required to reach criterion across multiple ASST stages, including the Rev (Fig. 5e_1; Vehicle = 16.7 vs. S-MK-26 = 11.7, p = 0.0221), IDS (15.6 vs 9.5, p = 0.0049), and EDS (24.3 vs 12.6, p < 0.0001). When examining its effect on attentional set formation, we found that S-MK-26 markedly blunted the IDS→EDS shift cost (Fig. 5e_2):

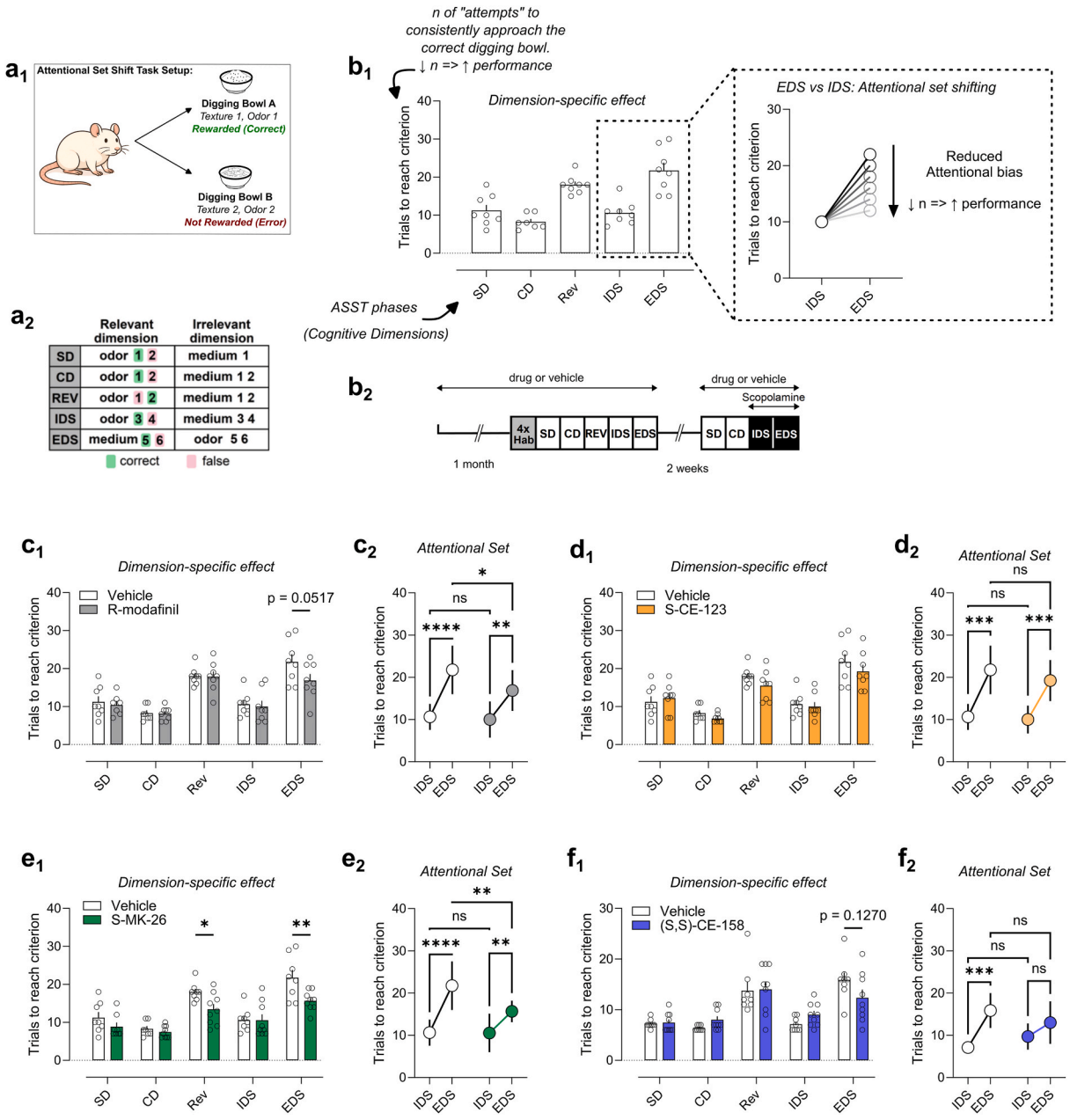

Fig. 4. Behavioral effects of modafinil analogs with improved in vitro pharmacology.

a_1) Schematics of the task; a_2) different cognitive dimensions assessed; b_1) example of ASST result output: the trials to reach criterion are quantified at each ASST phase. Over several discriminations (SD → CD → IDS), rats gradually form a cognitive bias: "attentional set". During the EDS, performance worsens, because the animal must abandon the set and switch to a new dimension; b2) Timeline of the ASST experiment; c) R-modafinil; c1) Dimension-specific effects of R-modafinil vs Vehicle: two-way mixed-effects ANOVA with main affect on test $F(4, 54) = 33.19$, $p < 0.0001$, Šídák's multiple comparisons test for EDS Vehicle vs R-modafinil $p = 0.0517$, for all the other tests $p > 0.05$; c2) R-modafinil effect on the attentional set-shifting: two-way mixed-effects ANOVA with main effect on test $F(1, 14) = 41.26$, $p < 0.0001$, Uncorrected Fisher's LSD for EDS Vehicle vs R-modafinil $p = 0.0424$, for Vehicle IDS vs EDS $p < 0.0001$, for R-modafinil IDS vs EDS $p = 0.0038$; d) S-CE-123; d1) Dimension-specific effects of S-CE-123 vs Vehicle: two-way mixed-effects ANOVA with main affect on test (IDS vs EDS) $F(4, 55) = 39.35$, $p < 0.0001$, for all test Šídák's multiple comparisons test Vehicle vs S-CE-123 $p > 0.05$; - d_2) S-CE-123 effect on the attentional set-shifting: two-way mixed-effects ANOVA with main affect on test (IDS vs EDS) $F(1, 14) = 46.46$, $p < 0.0001$; but not on compound $F(1, 14) = 0.9472$, $p = 0.3470$; Uncorrected Fisher's LSD for Vehicle IDS vs EDS $p = 0.0001$, S-CE-123 IDS vs EDS $p = 0.0006$; for IDS or EDS Vehicle vs S-CE-123 $p > 0.05$. *e) S-MK-26;* e_1) Dimension-specific effects of S-MK-26 vs Vehicle: two-way mixed-effects ANOVA with main affect on test $F(4, 58) = 33.52$, $p < 0.0001$, animals $F(1, 15) = 6.914$, $p = 0.019$, and test x animals $F(4, 58) = 2.752$, $p = 0.0364$. Šídák's multiple comparisons for ASST tests comparing Vehicle vs S-MK-26: Rev $p = 0.0480$, EDS $p = 0.0035$, for all the other tests $p > 0.05$; e_2) S-MK26 effect on the attentional set-shifting: two-way mixed-effects ANOVA: main affect on test (IDS vs EDS) $F(1, 15) = 43.19$, $p < 0.0001$; but not on compound $F(1, 15) = 3.738$, $p = 0.0723$, main effect on interaction test x compound $F(1, 15) = 5.925$, $p = 0.0279$; Uncorrected Fisher's LSD for EDS Vehicle vs. S-MK-26 $p = 0.0051$, Vehicle IDS vs EDS <0.0001, S-MK-26 IDS vs EDS 0.0087; for IDS Vehicle vs S-MK-26 $p > 0.05$. *f) (S,S)-CE-158;* f_1)Dimension-specific effects of (S,S)-CE-158 vs Vehicle: two-way mixed-effects ANOVA with main affect on test $F(4, 75) = 20.54$, $p < 0.0001$. Šídák's multiple comparisons for ASST tests comparing Vehicle vs (S,S)-CE-158: all the tests $p > 0.05$; - f_2) (S,S)-CE-158 effect on the attentional set-shifting: two-way mixed-effects ANOVA with main affect on test (IDS vs EDS) $F(1, 16) = 25.21$, $p = 0.0001$; but not on compound $F(1, 16) = 0.01328$, $p = 0.9097$, main effect on interaction test x compound $F(1, 16) = 5.157$, $p = 0.0373$;, Uncorrected Fisher's LSD for Vehicle IDS vs EDS $p = 0.0002$, while for both IDS and EDS Vehicle vs. (S,S)-CE-158 and for (S,S)-CE-158 IDS vs EDS $p > 0.05$.

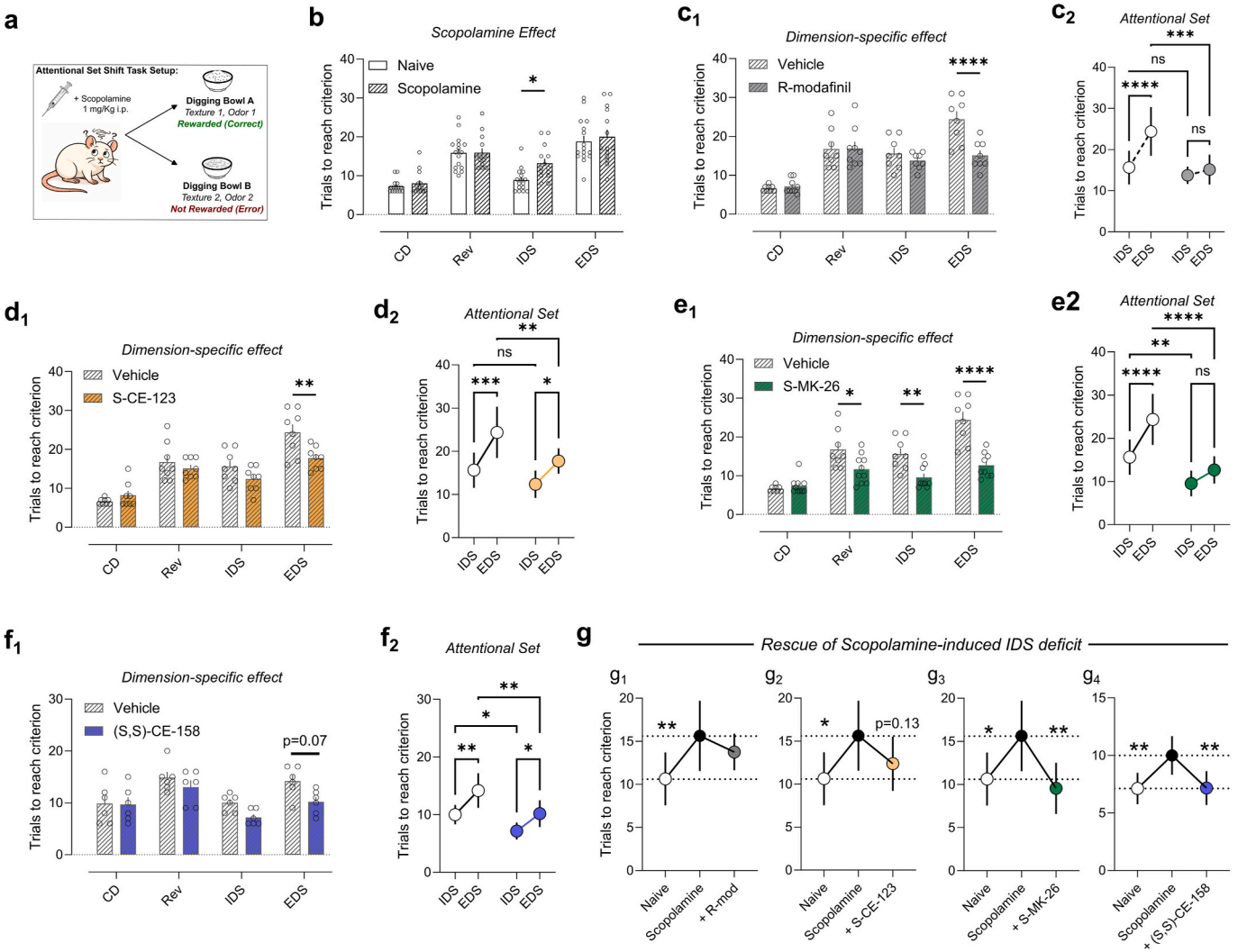

Fig. 5. Scopolamine-induced ASST deficits and behavioral rescue by the improved cognitive enhancers;
a) Schematics of the experiment; b) Scopolamine-induced ASST deficit: two-way mixed-effects ANOVA with main affect on test F (2.227, 61.62) = 57.95, $p < 0.0001$. Šídák's multiple comparisons test for IDS Vehicle vs Scopolamine $p = 0.0162$, for all the other tests $p > 0.05$; c) *R-modafinil;* c_1) Dimension-specific effects of R-modafinil vs Vehicle: two-way mixed-effects ANOVA with main affect on test F (3, 43) = 48.67, $p < 0.0001$, and interaction between test and treatment F (3, 43) = 8.014, $p < 0.0001$, Šídák's multiple comparisons test for EDS Vehicle vs R-modafinil $p < 0.0001$, for all the other tests $p > 0.05$; c_2) R-modafinil effect on the attentional set-shifting: two-way mixed-effects ANOVA with main effect on test F (1, 14) = 19.98,$p = 0.0005$, treatment F (1, 14) = 10.24, $p = 0.0064$, and interaction test x treatment F (1, 14) = 10.60, $p = 0.0057$, Uncorrected Fisher's LSD for EDS Vehicle vs R-modafinil $p = 0.0001$, for Vehicle IDS vs EDS $p < 0.0001$; in case of IDS Vehicle vs. R-modafinil and R-modafinil IDS vs EDS $p > 0.05$; *d) S-CE-123;* d_1) Dimension-specific effects of S-CE-123 vs Vehicle: two-way mixed-effects ANOVA with main affect on test (IDS vs EDS) F (3, 42) = 44.86, $p < 0.0001$, treatment F (1, 14) = 4.411, $p = 0.0543$, and interaction test x treatment F (3, 42) = 4.165, $p = 0.0114$; Šídák's multiple comparisons test for EDS Vehicle vs S-CE-123 $p = 0.0033$, for all the other ASST phases $p > 0.05$; - d_2) S-CE-123 effect on the attentional set-shifting: two-way mixed-effects ANOVA with main affect on test (IDS vs EDS) F (1, 14) = 26.39, $p = 0.0002$, on treatment F (1, 14) = 9.842, $p = 0.0073$; but not interaction F (1, 14) = 1.507, $p = 0.2399$. Uncorrected Fisher's LSD test for EDS Vehicle vs. S-CE-123: $p = 0.0037$, Vehicle IDS vs EDS: $p = 0.0005$, S-CE-123 IDS vs EDS $p = 0.0152$; in case of IDS Vehicle vs. R-modafinil and R-modafinil IDS vs EDS $p > 0.05$. *e) S-MK-26;* e_1) Dimension-specific effects of S-MK-26 vs Vehicle: two-way mixed-effects ANOVA with main affect on test F (3, 46) = 36.28, $p < 0.0001$, animals F (1, 16) = 23.73, 0.0002, and test x animals F (3, 46) = 10.47, p= <0.0001. Šídák's multiple comparisons for ASST tests comparing Vehicle vs S-MK-26: Rev $p = 0.0221$, IDS $p = 0.0049$, EDS $p < 0.0001$, CD $p > 0.05$; e_2) S-MK26 effect on the attentional set-shifting: two-way mixed-effects ANOVA: main affect on test (IDS vs EDS) F (1, 15) = 29.36, $p < 0.0001$; compound F (1, 15) = 28.06, $p < 0.0001$, and interaction test x compound F (1, 15) = 6.636, $p = 0.0211$; Uncorrected Fisher's LSD: IDS Vehicle vs. S-MK-26 $p = 0.0050$; EDS Vehicle vs. S-MK-26 $p < 0.0001$, Vehicle IDS vs EDS $p < 0.0001$, S-MK-26 IDS vs EDS $p = 0.0559$; *f) (S,S)-CE-158;* f_1)Dimension-specific effects of (S,S)-CE-158 vs Vehicle: two-way mixed-effects ANOVA with main affect on test F (3, 30) = 11.59, $p < 0.0001$. Šídák's multiple comparisons for ASST tests comparing Vehicle vs (S,S)-CE-158: all the tests $p > 0.05$, EDS,$p = 0.0740$; - f_2) (S,S)-CE-158 effect on the attentional set-shifting: two-way mixed-effects ANOVA with main affect on test (IDS vs EDS) F (1, 10) = 20.59, $p = 0.0011$, treatment F (1, 10) = 11.85, $p = 0.0063$, but no interaction test x compound F (1, 10) = 0.5457, $p = 0.4771$; Uncorrected Fisher's LSD: IDS Vehicle vs. (S,S)-CE-158 $p = 0.0371$; EDS Vehicle vs. (S,S)-CE-158 $p = 0.0050$, Vehicle IDS vs EDS $p = 0.0039$, (S,S)-CE-158 IDS vs EDS $p = 0.0228$; g) *Rescue of the scopolamine-mediated IDS deficit* in the ASST by cognitive enhancers. Statistics: one-way ANOVA and Dunnett's multiple-comparison test; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$: i) R-modafinil - One-way ANOVA treatment effect F (2, 21) = 4.950, $p = 0.0173$; Dunnett's multiple-comparison test Scopolamine vs. Naïve: $p = 0.0099$, vs + R-modafinil $p = 0.4108$; ii) S-CE-123 - One-way ANOVA treatment effect F (2, 21) = 4.238, $p = 0.0284$; Dunnett's multiple-comparison test Scopolamine vs. Naïve: $p = 0.0172$, vs + S-CE-123 = 0.1336; iii) S-MK-26 - One-way ANOVA treatment effect F (2, 22) = 7.546, $p = 0.0032$; Dunnett's multiple-comparison test Scopolamine vs. Naïve: p = 0. 0.0142, vs + S-MK-26 $p = 0.0024$; iv) (S,S)-CE-158. - One-way ANOVA treatment effect F (2, 17) = 7.730, $p = 0.0041$; Dunnett's multiple-comparison test Scopolamine vs. Naïve: $p = 0.0044$, vs + (S,S)-CE-158 $p = 0.0080$.

whereas Vehicle-treated rats showed a clear cognitive bias (IDS vs EDS, $p < 0.0001$), this difference was strongly attenuated in S-MK-26–treated animals (IDS vs EDS $p = 0.0559$).

In contrast, (S,S)-CE-158 produced only a modest reduction in trials to criterion across ASST stages, including the EDS (Fig. 5f$_1$; Vehicle = 14.2 vs (S,S)-CE-158 = 10.2, $p = 0.074$). Nevertheless, (S,S)-CE-158 significantly reduced the overall IDS→EDS shift cost (Fig. 5f$_2$; Vehicle IDS vs EDS, $p = 0.0039$; (S,S)-CE-158 IDS vs EDS, $p = 0.0228$), indicating a qualitatively similar effect on attentional set formation to that observed with S-MK-26.

Given the selective effect of scopolamine treatment of the IDS (i.e. the ASST phase evaluating the consolidation of an attentional set), we also evaluated the impact of the compounds on the IDS only. In this specific case, R-modafinil did not show any effect on the rescue (Fig. 5g$_1$, $\Delta_{\text{Scopol-Naïve}} = 0.4688$, $p = 0.0099$; $\Delta_{\text{Scopol-R-mod}} = 0.1763$, $p = 0.4108$), while S-CE-123 show only a partial rescue (Fig. 5g$_2$, $\Delta_{\text{Scopol-Naïve}} = 0.4688$, $p = 0.0172$; $\Delta_{\text{Scopol-S-CE123}} = 0.3050$, $p = 0.1336$). Instead, a full rescue of performances was present for both slow binding-kinetic inhibitors S-MK-26 (Fig. 5g$_3$, $\Delta_{\text{Scopol-Naïve}} = 0.4688$, $p = 0.0142$; $\Delta_{\text{Scopol-S-MK-26}} = 0.5722$, $p = 0.0024$) and (S,S)-CE-158 (Fig. 5g$_4$, $\Delta_{\text{Scopol-Naïve}} = 0.4025$, $p = 0.0044$; $\Delta_{\text{Scopol-S-CE158}} = 0.3967$, $p = 0.0080$). These effects are consistent with the *in vitro* k_{off} values measured in patch-clamp experiments (Fig. 1f).

4. Discussion

Drugs that potently interact with DAT have historically been associated with a high potential for abuse (Ritz et al., 1987), particularly those that exhibit greater potency at DAT compared to SERT (Wee et al., 2005). Indeed, the preference for DAT over SERT, i.e., the DAT/SERT selectivity ratio, is regularly used as a predictive indicator of the abuse liability of a given drug (Bauer et al., 2013; Luethi and Liechti, 2020). However, several high affinity and DAT-selective inhibitors such as benztropines, rimcazole, GBR12909, and related analogs do not induce robust reinforcing and stimulatory effects (Newman et al., 2021; Newman and Kulkarni, 2002; Reith et al., 2015). On the other hand, cocaine, which induces reinforcing and stimulatory effects, does not present with a high DAT/SERT selectivity ratio. Hence, the molecular basis for the differences in behavioral effects across different DAT inhibitors is still not completely understood, and numerous hypotheses have been put forward (Reith et al., 2015). In addition, most preclinical research on psychostimulants has focused on modelling abuse liablity, with little insights into their potential therapeutic effects, such as enhancing energy and cognition (Wood et al., 2013). The *in vitro* pharmacology of drugs is typically assessed under thermodynamic equilibrium conditions. Yet, in the human body, drug-target interactions are influenced by the constant flux of fluids and a variety of physiological processes. Hence, the *in vitro* effects of a given drug may not fully predict *in vivo* activity (Copeland, 2016; Copeland et al., 2006). In the past, it was suggested that the DAT-selective benztropine analog JHW007 could antagonize the reinforcing effects of cocaine by slowly occupying DAT in the CNS (Desai et al., 2005; Velázquez-Sánchez et al., 2010). Recently, we observed that the rate of unbinding of compounds at DAT predicts the length of stimulatory effects across different psychostimulants, indicating that binding kinetics can influence behavioral properties of DAT ligands (Niello et al., 2023). Altogether, the collected evidence suggests that binding kinetics may have an important role in the pharmacological manipulation of DAT. Furthermore, establishing kinetic-activity relationships for DAT ligands may help develop inhibitors with improved pharmacological properties and therapeutic outcomes.

In the present study, we delved deeper into the role of psychostimulants and DAT in complex behaviors. In particular, we looked at analogs of R-modafinil - a DAT inhibitor that shares similar neurochemical effects with other psychostimulants such as cocaine and amphetamine, show much lower abuse liability, while maintaining beneficial effects (Mereu et al., 2013). Furthermore, modafinil was found capable of improving cognitive performance in healthy and sleep-deprived human subjects (Makris et al., 2007; Wesensten, 2006), and showed promising results in schizophrenia patients when used in combination with antipsychotic treatments (Farrow et al., 2006; Rosenthal and Bryant, 2004). Despite the positive therapeutic results, the molecular pharmacology of modafinil is still not fully elucidated (Mereu et al., 2013). Different analogs were developed in the attempt to improve modafinil's therapeutic effects (Tanda et al., 2021). In our study, we observed that the improvement in the equilibrium affinity from μM to sub-μM of different modafinil analogs is associated with their slower k_{off} at DAT (Fig. 1f). We found that replacing the terminal amide of R-modafinil with a thiazole, in combination with substitutions on the bis-phenyl ring system, plays a role in binding kinetics (Fig. 1f). However, in contrast to pyrovalerone-based stimulants (Niello et al., 2023), we observed that all the tested analogs likely depend on the interaction with a single state of the DAT transport cycle, suggesting a more homogeneous molecular pharmacology for modafinil analogs compared to pyrovalerone analogs. In our study, we did not assess if the tested inhibitors also stabilize the inward-facing conformation of DAT as reported already for R-modafinil (Loland et al., 2012), and therefore, we cannot conclude if the tested inhibitors might be used for state-dependent modulation of DAT as reported for GBR12909 and benztropine (Li et al., 2024). In addition, R-modafinil analogs compared in this study are all thiazole derivatives, but their sulfoxide stereochemistry is S-. Thus, in contrast to R-modafinil, it is the opposite enantiomer that exhibits higher DAT affinity. Given the critical role of sulfoxide configuration in DAT binding for R-modafinil and related compounds, further atomistic studies will be necessary for elucidating the binding interactions of these analogs. Interestingly, the optimized DAT inhibitors produced stronger cognitive effects than R-modafinil, despite some differences between the optimized compounds were also present. It could be that the slow dissociation rate could be achieved via the establishment of occluded intermediates, as reported already for LSD in the case of 5HT2b and 5HT2a receptors (Kim et al., 2020; Wacker et al., 2017), or by stabilizing the inward-open state as established in the case of GBR12909 and benztropines (Li et al., 2024). The molecular consequences of a long residence time at DAT are still enigmatic. Further studies will be needed to clarify this point. However, our study revealed clear neurochemical and behavioral differences between inhibitors exhibiting slow versus fast binding kinetics.

We have carried out a pharmacokinetics study to evaluate if, following oral administration, the compounds of interest can cross the blood-brain barrier and efficiently enter the CNS. While R-modafinil showed a brain-to-plasma ratio <1, all the other compounds showed a brain-to-plasma ratio ≥ 1, indicating an overall good brain partitioning of the new compounds compared to R-modafinil. Moreover, both S-MK-26 and (S,S)-CE-158 showed a brain-to-plasma ratio close to 2, indicating that they are likely to be accumulated in the brain following oral administration. It is worth mentioning that our study did not assess the free-drug concentration for S-MK-26 and (S,S)-CE-158, as was done for R-modafinil and S-CE-123 (Spreitzer et al., 2023), which limits our ability to determine the true drug exposure (Deo et al., 2013; Kulkarni et al., 2016; Loryan et al., 2022).

We then evaluated the effects of the compounds in the ASST, a task with strong predictive validity for set formation and set shifting (Goetghebeur and Dias, 2009; Nagahara et al., 2010; Rock et al., 2014). In healthy rats, all DAT inhibitors tended to reduce trials to criterion at EDS, indicating a general facilitation of cognitive flexibility. The same pattern was even more consistent in scopolamine-treated rats, in which every compound reduced the EDS shift cost. By contrast, scopolamine—a non-selective muscarinic antagonist commonly used to model amnestic and cognitive-decline phenotypes—primarily impaired IDS performance, and rescue of IDS (i.e., attentional set formation) was most evident for the optimized DAT inhibitors S-MK-26 and (S,S)-CE-158, whereas R-modafinil and S-CE-123 showed weaker or no IDS rescue.

This pattern is internally consistent with the stability–flexibility trade-off (Musslick et al., 2018; Musslick and Cohen, 2021; Shafiei et al., 2019). Set formation (IDS) demands strong and sustained dopaminergic engagement to stabilize task-relevant representations, whereas set shifting (EDS) can be facilitated by comparatively modest DA elevations that bias behavior away from perseveration and toward exploration. In line with this interpretation, our PK analysis indicates that S-MK-26 and (S,S)-CE-158 achieve higher brain exposure and longer effective target engagement at DAT, whereas R-modafinil and S-CE-123 show lower or shorter-lived CNS penetration. Thus, under scopolamine, only compounds with robust cortical exposure and sufficient DAT residence time reliably restore attentional stability (IDS), while even weaker DAT inhibitors are sufficient to promote flexibility (EDS).

Mechanistically, S-MK-26 and (S,S)-CE-158 not only show improved DAT affinity and selectivity over SERT/NET, but also longer DAT residence time, collectively supporting an on-target dopaminergic mechanism for the cognitive benefits observed. Taken together, these results reconcile the apparent discrepancy: DAT inhibition can promote set shifting even when cholinergic tone is low, but restoring attentional set formation under cholinergic disruption requires higher central dopaminergic load and sustained DAT engagement.

Our results expand previous findings on S-MK-26 (Kouhnavardi et al., 2022), and (S,S)-CE-158 (Lubec et al., 2021), and further support the value of testing DAT-inhibitors in animal models of neurodevelopmental disorders (Santoni and Pistis, 2024).

In conclusion, our study demonstrates that designing modafinil analogs with high DAT selectivity and longer residence times enhances their cognitive effects, indicating that these actions are primarily mediated by dopamine rather than other monoaminergic systems, as previously suggested (Mignot et al., 1994). Consistent with this, (S,S)-CE-158 showed no off-target activity across a broad range of GPCRs and kinases (Lubec et al., 2021), suggesting that any contribution of other neurotransmitter systems *in vivo* (Gerrard and Malcolm, 2007; Reith et al., 2015) is likely indirect (Devoto et al., 2020; Frau et al., 2022). Furthermore, our findings highlight that optimizing DAT binding kinetics profoundly shapes the molecular, neurochemical, and behavioral properties of these inhibitors. Taken together, these results underscore the importance of detailed assessments of drug–DAT binding kinetics to inform the rational design of novel compounds with potentially improved clinical utility.

Declaration of generative AI and AI-assisted technologies in the manuscript preparation process

During the preparation of this work, the author(s) used ChatGPT and Microsoft 365 Copilot to improve text clarity and readability. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the published article.

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CRediT authorship contribution statement

Marco Niello: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Supervision, Visualization, Writing – original draft, Writing – review & editing. **Predrag Kalaba:** Investigation, Project administration, Writing – review & editing. **Anita Cybulska-Klosowicz:** Formal analysis, Investigation, Methodology. **Michael Kirchhofer:** Investigation, Methodology. **Iva Spreitzer:** Investigation, Methodology. **Markus Spreitzer:** Formal analysis, Investigation. **Judith Wackerlig-Damle:** Supervision. **Michele Santoni:** Data curation, Investigation, Methodology, Writing – review & editing. **Claudia Sagheddu:** Conceptualization. **Simone B. Sartori:** Conceptualization. **Karl Ebner:** Conceptualization. **Jana Lubec:** Project administration, Resources. **Ahmed M. Hussein:** Investigation. **Tamara Stojanovic:** Investigation. **Nicolas Singewald:** Conceptualization. **Marco Pistis:** Project administration, Resources, Writing – review & editing. **Gert Lubec:** Project administration, Resources. **Harald H. Sitte:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuropharm.2026.110969.

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