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REGULAR RESEARCH ARTICLE

Converging Evidence on D-Amino Acid Oxidase–Dependent Enhancement of Hippocampal Firing Activity and Passive Avoidance Learning in Rats

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Abstract

Background: N-methyl-D-aspartate (NMDA) receptor activation requires the binding of a co-agonist on the glycine-binding site. D-serine is the main endogenous co-agonist of NMDA receptors, and its availability significantly depends on the activity of the metabolic enzyme D-amino acid oxidase (DAAO). Inhibition of DAAO increases the brain levels of D-serine and modulates a variety of physiological functions, including cognitive behavior.
Methods: Here, we examined the effects of a novel 4-hydroxypyridazin-3(2H)-one derivative DAAO inhibitor, Compound 30 (CPD30), on passive avoidance learning and on neuronal firing activity in rats.
Results: D-serine administration was applied as reference, which increased cognitive performance and enhanced hippocampal firing activity and responsiveness to NMDA after both local and systemic application. Similarly to D-serine, CPD30 (0.1 mg/kg) effectively reversed MK-801–induced memory impairment in the passive avoidance test. Furthermore, local iontophoretic application of CPD30 in the vicinity of hippocampal pyramidal neurons significantly increased firing rate and enhanced their responses to locally applied NMDA. CPD30 also enhanced hippocampal firing activity after systemic administration. In 0.1- to 1.0-mg/kg doses, CPD30 increased spontaneous and NMDA-evoked firing activity of the neurons. Effects of CPD30 on NMDA responsiveness emerged faster (at 10 minutes post-injection) when a 1.0-mg/kg dose was applied compared with the onset of the effects of 0.1 mg/kg CPD30 (at 30 minutes post-injection).
Conclusions: The present results confirm that the inhibition of DAAO enzyme is an effective strategy for cognitive enhancement. Our findings further facilitate the understanding of the cellular mechanisms underlying the behavioral effects of DAAO inhibition in the mammalian brain.

Key Words: DAAO, D-serine, NMDA receptor, hippocampus, memory

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Significance Statement

D-serine is an amino acid that has an important role in the function of the brain. D-serine is required for the activation of neurons via the NMDA subtype of glutamate receptors. The available amount of D-serine is dependent on the enzymatic activity of D-amino acid oxidase (DAAO). Inhibitors of DAAO are reported to increase the brain concentration of D-serine, improve neuronal communication, and alleviate the symptoms of neuropsychiatric states such as depression and cognitive impairment. Here, we tested the effects of a novel DAAO inhibitor (Compound 30) on neuronal activity and on cognitive performance in rats. Our results showed that Compound 30 increased activity and excitability of hippocampal neurons and improved memory in an aversive conditioning task. In conclusion, our findings uncover a relationship between the neural and behavioral action of DAAO inhibition. Our research facilitates the understanding of mechanisms of action of DAAO inhibitors and suggests further therapeutic applications.

Introduction

N-methyl-D-aspartate receptors (NMDARs) are widely expressed in the central nervous system and play a critical role in structural and functional synaptic plasticity. Hence, the dysfunction of NMDARs is involved in numerous neurological disorders such as cognitive disorders, schizophrenia, chronic pain, and amyotrophic lateral sclerosis (Paoletti and Neyton, 2007). Activation of NMDARs requires 3 separate actions: (1) binding of glutamate to its GluN2 subunit; (2) post-synaptic depolarization, which relieves the Mg^{2+} blockade of the ion channel; and (3) glycine or D-serine binding on the glycine modulatory site (GMS) of the GluN1 subunit (Paoletti and Neyton, 2007). Although in earlier studies glycine was thought to be the main substrate of GMS, more recent studies emphasize the role of extracellular D-serine in the modulation of GMS under physiological circumstances. It is well accepted that synaptic NMDARs are mostly gated by D-serine, whereas extrasynaptic receptors are gated by glycine (Papouin et al., 2012; Sullivan and Miller, 2012). Several studies implicated an age-related decline in the activation of NMDARs, which can be associated with the decrease of D-serine levels in the hippocampus (Junjaud et al., 2006; Potier et al., 2010). Also, the GMS is a promising pharmacological target to enhance NMDAR activity, while the direct targeting of the glutamate-binding site with agonists may cause Ca^{2+}-induced neurotoxicity (Choi, 1988). The GMS is not saturated in vivo, which further supports its potential as a therapeutic target (Bergeron et al., 1998).

D-serine exhibits poor brain penetration; only high doses of i.p. injections of D-serine raises its concentrations in the brain of adult rats (Hashimoto et al., 1993; Takahashi et al., 2002). There are also safety concerns that high concentrations of D-serine can cause nephrotoxicity related to its metabolism (Carone and Ganote, 1975). One of the mechanisms limiting the brain concentration of D-serine is the D-amino acid oxidase (DAAO) enzyme that is the main catabolic enzyme to degrade D-serine. In the mammalian brain, it has been confirmed with in situ hybridization that DAAO, both in rats and humans, shows relatively high expression throughout the brain, including the neocortex and the hippocampus, which is crucial for the formation and maintenance of memory function (Moreno et al., 1999; Verrall et al., 2007). Preclinical findings regarding the enhancement of NMDAR through the modulation of the GMS suggested that DAAO inhibitors might be useful as novel therapeutics to treat various psychiatric and neurocognitive disorders (Verrall et al., 2010). DAAO inhibitors were first developed as potential antipsychotic agents, and only some of them were investigated as cognitive enhancers (Smith et al., 2010). Sodium benzoate was one of the first compounds described as a selective inhibitor of the DAAO enzyme. Despite its moderate binding to the enzyme and its weak inhibitory effect, the compound was effective in alleviating psychosis-like symptoms in animal models of schizophrenia (Sacchi et al., 2013). Sodium benzoate, as a DAAO inhibitor, was under clinical investigation not only in schizophrenia (Lin et al., 2018) but also in mild cognitive impairment and Alzheimer's disease (Lin et al., 2014). In addition, sodium benzoate, as an add-on therapy, effectively improved cognitive function of schizophrenia patients (Lane et al., 2013) and ameliorated behavioral and psychological symptoms of dementia (Lin et al., 2019, 2020).

Data regarding the beneficial effects of novel and more efficacious DAAO inhibitors on cognitive functions are controversial. Hondo et al. (2013) published the first positive results demonstrating the procognitive effects of a novel selective DAAO inhibitor. In their report, the 4-hydroxypyridazin-3(2H)-one derivative Compound 30 (CPD30) showed inhibitory action for human, mouse, and rat DAAOs in the nanomolar range and was found to be effective against MK-801–induced cognitive deficits in mice at very low doses. Hopkins et al. (2013) further supported the contribution of DAAO inhibition on cognitive performance using the Sunovion compound SUN in rodent models. In contrast, previously synthesized DAAO inhibitors resulted in significant enzyme inhibition both in vitro and ex vivo (Lange et al., 2011) but failed to induce cognitive enhancement in rat models (Smith et al., 2009; Strick et al., 2011).

In the present study, we intended to compare the efficacy of the DAAO inhibitor CPD30 with the effect of externally applied D-serine in a drug-induced memory impairment model in rats. To further explore the underlying system-level changes, we investigated the modulatory effects of locally or systemically applied CPD30 and D-serine on the spontaneous firing activity and NMDA responsiveness of rat hippocampal neurons in vivo.

Methods

Ethical Statement

The study was conducted in full compliance with the European Union legislation Directive 2010/63/EU and national legal requirements of the decree no. 40/2013. (II.14.) "On animal experiments" issued by the Ministry of Agriculture of the Government of Hungary. All experimental procedures were reviewed and approved by the Local Animal Care and Use Committees of Gedeon Richter Pharmaceutical Plc. (PE/EA/2885–6/2016) and the University of Pécs (BA02/2000–80/2017).

Behavioral Assessments in the Passive Avoidance Paradigm

Animals and Housing—Fifty male Wistar-Han rats were used for the investigation of the behavioral effects of D-serine and another 50 animals for the studies with CPD30. Animals were

purchased from a commercial vendor (Toxicoop, Hungary) and weighed 160–180 g at the beginning of the experiments. The rats were group-housed (4 per cage) and kept in the animal facility of Gedeon Richter Plc. (Budapest, Hungary) with a 12 hour-light/-dark cycle for 10 days prior to testing. Ambient temperature was maintained at 22°C ±2°C with 40%–50% relative humidity. Food and water were freely available for the animals.

***Passive Avoidance Testing*—**Cognitive performance of the rats was tested using a conventional 2-compartment (light/dark) passive avoidance testing apparatus and software manufactured by TSE Systems (Bad Homburg, Germany). The experiments were carried out at Gedeon Richter Plc. according to Kaada et al. (1962). The experiments were conducted on 3 consecutive days. On the first day ("habituation"), the animals were acclimatized to the testing apparatus and the noise generated by operating the door between the 2 compartments of the apparatus. The animals were put into the bright compartment with closed door, and after 30 seconds the door opened, allowing the animals to move to the naturally more comfortable dark compartment. After 30 seconds elapsed, the animals were taken out of the apparatus and were put back in their homecage. On the second day ("training"), the animals were put in the bright compartment with the door open. When the animals entered the dark compartment, the door was closed, and they received a mild foot-shock (0.7 mA for 10 seconds). After the foot-shock, the animals were left in the dark compartment for an additional 30 seconds, allowing them to associate the aversive unconditioned stimulus (foot-shock) with the dark compartment of the apparatus (conditioned stimulus). On the third day ("retention"), the animals were placed again into the bright compartment with the door open. The time elapsed until the transition to the dark compartment was measured as the readout of memory performance. When the animals entered the dark compartment, they were taken out from the apparatus and the session ended. The cut-off time was set to 300 seconds. If an animal did not cross the door, the maximum 300 seconds was recorded as latency to dark compartment transition and a note was made that the animal did not enter the dark compartment.

***Pharmacological Treatments*—**Treatments were applied on the second day before the training session of the passive avoidance experiment. Comparisons were made in a between-subject design (i.e., each animal was tested only once in the passive avoidance task). All groups except the vehicle (control) group were injected with 0.25 mg/kg NMDAR antagonist MK-801 (Sigma-Aldrich) i.p. 30 minutes prior to testing to induce transient amnesia for the training session. The effects of D-serine and CPD30 were tested in 2 separate experiments. In the first experiment, D-serine was dissolved in a mixture of 5% Tween 80 and phosphate buffered saline and was administered subcutaneously (s.c.) 60 minutes prior to testing in 320-, 640-, and 1280-mg/kg doses (DSer320, DSer640 and DSer1280, respectively). D-serine injections were substituted with the solvent of the drug in the vehicle and MK-801 groups. Thus, in the first experiment, the following 5 experimental groups were compared (n = 10 in each group): vehicle, MK-801, MK-801 plus D-serine at 320-, 640-, and 1280-mg/kg doses, respectively. In the second experiment, CPD30 was dissolved in 10% PEG-400 (in phosphate buffered saline), and was administered per os 60 minutes prior to testing. CPD30 administrations were substituted with the solvent of the compound in the vehicle and MK-801 groups. Thus, in the second experiment, the following 5 experimental groups were compared (n = 10 in each group): vehicle, MK-801, and MK-801 plus CPD30 at 0.1 mg/kg, 0.3 mg/kg, and 1 mg/kg, respectively.

Statistical Analysis

Passive avoidance data were analyzed in a Cox proportional hazard model (Jahn-Eimermacher et al., 2011) in R version 3.6.2 under RStudio v1.2.5033 using "survival" and "survminer" packages (Kassambara et al., 2019; R Core Team, 2019; RStudio Team, 2019; Therneau and Grambsch, 2000). The Cox model is nonparametric and requires only that probability (hazard) of the transition to the dark compartment remains constant during the experimental period (0–300 seconds from start). The model calculates hazard ratio (effect size), which measures the relative risk that the event will occur in a treatment compared with a reference treatment. The MK-801 group was chosen as the reference treatment with a hazard ratio of 1. A higher hazard ratio represented a greater probability of entering the dark zone (worse memory performance), while a hazard ratio <1 represented a lower probability of entering the dark zone (better memory performance).

In Vivo Electrophysiology

***Animals and Surgery*—**Altogether 94 adult male Wistar-Han rats weighing 185 to 350 g were used in the experiments. Between arrival and experiments, rats were kept in a conventional animal house under the standard 12-hour light cycle from 6 AM to 6 PM with controlled temperature and humidity. Water and standard laboratory rat chow were available ad libitum. Surgical preparations were carried out as described previously (Bali et al., 2017). Briefly, rats were initially anesthetized with an i.p. injection of chloral hydrate (400 mg/kg body weight), and stable anesthesia was further maintained by continuous i.v. administration of the anesthetic via a jugular vein cannula (chloral hydrate, 100 mg/kg/h initially, later adjusted if needed). Stereotaxic surgery was performed in a stereotaxic frame to allow the insertion of a multi-barrel carbon fiber microelectrode (Carbostar-3, -4 or -7S, Kation Scientific Ltd., Minneapolis, MN) (Budai et al., 2010) into the CA1 region of the dorsal hippocampus (in conformity with the rat brain atlas by Paxinos and Watson [2014]: AP 3.4–5.1, ML 1.6–3.3 from bregma, and DV 1.7–3.9 from dura). At the end of the recording sessions, rats were killed by i.v. administration of an overdose of pentobarbital (Euthanimal 40%, Alfasan, the Netherlands).

***In Vivo Electrophysiological Recording and Microiontophoresis*—**Firing activity of CA1 neurons was recorded extracellularly through the central carbon fiber of the multi-barrel microelectrode. The electrophysiological signal was amplified and band-pass filtered between 400 and 3500 Hz by analog electrophysiological amplifiers (BioAmp, Supertech Ltd., Pécs, Hungary; NeuroLog, Digitimer Ltd., Welwyn Garden City, UK). The signal was digitized at 25 kHz with an analog-to-digital converter unit (CED Power 1401) using Spike2 software (both manufactured by Cambridge Electronic Design Ltd., Cambridge, UK). During recordings, iontophoretic drug delivery (Neurophore BH-2 System, Medical System Corp., Greenvale, NY) was performed using the glass pipettes surrounding the central carbon-fiber recording channel of the microelectrode. Between individual drug administrations, a low retention current with the opposite charge was applied onto the microiontophoresis pipettes to avoid leakage of the compounds. One of the glass capillaries of the multi-barrel electrode was filled with 50 mM NMDA (Sigma-Aldrich). In experiments investigating the local effects of the test compounds, additional micropipettes were filled with one of the following test compounds (manufacturer, pipette

concentrations, and microiontophoretic ejection currents are in parentheses): D-serine (Sigma-Aldrich, 50 mM in 0.003 N NaOH: −5 to −100 nA), CPD30 (synthesized at Orion Pharma, Finland, and supplied by Gedeon Richter Plc., Hungary, 100 mM in 40% DMSO in 0.12 N NaOH, −5 to −110 nA). Analysis of electrophysiological data was performed offline. Extracellular action potentials (spikes) were detected from the continuous waveform data (raw data) using the template matching algorithm of the Spike2 software (see above) applying a spike threshold of 5 times the root-mean-square of noise. Only those spike clusters that showed the typical characteristics of complex-spiking cells (putative pyramidal cells) on the basis of autocorrelogram analysis were included in further analyses (Csicsvari et al., 1998; Bali et al., 2014).

Investigation of Local Effects of the Test Compounds—Local effects of test compounds were evaluated using an experimental protocol similar to our recent publication (Bali et al., 2019). NMDA was periodically ejected for 5 seconds in every 2 minutes. After the optimal ejection current and a stable firing response of the neurons to NMDA were achieved, one of the test compounds was applied for 70 seconds, overlapping the next NMDA event in the last 10 seconds of the drug delivery. The application of drugs was repeated at least 2 times in a recording session with different ejection currents.

Investigation of Systemic Effects of Drugs—D-serine was dissolved in sterile saline (0.9% NaCl) to a concentration of 320 mg/mL and was applied at a 1280-mg/kg dose. CPD30 was dissolved in 10% PEG-400 and phosphate buffered saline to a concentration of 0.02 or 0.2 mg/mL and was applied at a 0.1- or 1.0-mg/kg dose, respectively. The experimental protocol was similar to that we used in our earlier publication (Bali et al., 2017). Similarly to the experiments studying local effects, NMDA was periodically ejected by negative constant current for 5 seconds every 2 minutes. After the firing responses of the neurons to NMDA were stable for 5 consecutive NMDA applications (10 minutes, control phase), D-serine or CPD30 was injected systemically via s.c. injection. After drug administration, the NMDA application was kept periodic throughout the entire recording session. Changes in spontaneous and NMDA-evoked firing activity were monitored for at least 1 hour after the systemic administration of the test compound.

Firing Activity Calculations—Spontaneous firing rate (Hz) in a given period was measured in a 60-second-long time window immediately before the next local NMDA administration. The NMDA-evoked firing rate was defined as the average firing rate during the given excitation event evoked by the iontophoresis of NMDA.

Local Effects of Test Compounds—Effects on spontaneous and NMDA-evoked firing rates were evaluated on a trial by trial basis. First, they were calculated during the iontophoresis of the test compounds. Next, we normalized the compound-induced firing rate to the corresponding pre-administration control value. The control value was the spontaneous firing rate 120 seconds before the drug administration and the mean of the NMDA-evoked firing rate in the 3 previous NMDA-evoked excitation peaks. The effects of the test compound in the given trial were determined as firing rate increase (normalized firing rate ≥1.2), decrease (≤0.80), or no effect (between 0.8 and 1.2) on both spontaneous and NMDA-evoked firing activity. To use neurons as the statistical unit for hypothesis testing, we determined the net effect and firing rate data neuron by neuron. Thus, the effect (increase or decrease) that occurred more frequently and was repeated at least once during the recording of 1 particular neuron was considered as the net response of the neuron. Furthermore, we also calculated mean raw and normalized firing rate values in spontaneous and NMDA-evoked firing conditions by averaging trial-wise data measured on the given neuron. To compare the magnitude of the effects evoked by the test compounds, the mean normalized firing rate on a given neuron was calculated from those trials in which the same as the typical net effect was observed.

Distribution of firing rate increasing and decreasing effects of a given compound in the given test condition (i.e., spontaneous or NMDA-evoked firing) was analyzed with the binomial test. The null hypothesis in the binomial test was that firing rate increasing and decreasing effects were distributed with equal probability. Thus, a significant result showed a definite firing rate increasing or decreasing effect of the test compound. Effects exerted by the test compounds on spontaneous and NMDA-evoked firing rate were also evaluated by comparing firing rate values before (control) and during the iontophoresis of the test compound using paired samples *t* test. For this purpose, we used the data from individual trials, weighted by the reciprocal of the number of trials in the given recording to ensure balanced involvement of each recording session in the final data pool and statistics. Additionally, we also compared efficacy of the test compounds. For this purpose, the direction of firing rate changes (increase, decrease, no change) after different treatments were evaluated according to the chi-squared distribution. Furthermore, the magnitude of the effects of test compounds was compared using the normalized firing rate with paired samples *t* test.

Systemic Effects of the Test Compounds—Pretreatment control data (T0) of spontaneous and NMDA-evoked firing rate in a given recording session were determined as the average of the last 3 firing rates before the administration of the test compounds. Then, effects of the test compounds as a function of time elapsed from the drug administration was investigated by calculating spontaneous and NMDA-evoked firing rate data at every 10 minutes (T10, T20, T30, T40, T50, T60) after the control measurement. At each measurement point, firing rate values were determined in 3 consecutive periods and averaged for further analyses.

Effectiveness of the test compounds on the spontaneous and NMDA-evoked firing rates was separately tested using linear mixed-effects models in IBM SPSS Statistics 25 (SPSS). In the linear mixed-effects model, neuron ID was used as a random intercept to take into account the correlation between repeated measurements. Significance (*P* values) corresponding to estimated marginal mean differences between post-injection time points (T10–T60) and the control measurement (T0) were corrected for multiple comparisons using Holm's method (Holm, 1979). Furthermore, efficacies of the test compounds were compared with the corresponding vehicle control by comparing the regression slopes of firing rate change after different treatments in contrast to the vehicle over the time course of the recording session. The slopes were compared using interaction terms of the following linear mixed-effects model:

$$\begin{aligned} FIRING_{ij} = {} & b_0 + b_1 \times TIME_{ij} + b_2 \times COMPOUND[1]_{ij} + b_3 \\ & \times COMPOUND[2]_{ij} + b_4 \times TIME_{ij} \times COMPOUND[1]_{ij} + b_5 \\ & \times TIME_{ij} \times COMPOUND[2]_{ij} + v_i + e_{ij} \end{aligned}$$

where FIRING is the resulted firing rate in Hz, TIME is a continuous predictor meaning the time elapsed from injection, COMPOUND[1] and COMPOUND[2] are the test compounds in

the given experiment (i.e., D-serine or CPD30 at 0.1 and 1.0 mg/kg), b_0-b_5 are the parameters to be estimated, v_i is the random intercept, and e is the error term. Indices correspond to the *i*-th subject and *j*-th repeated measurement. After finding a significant interaction of TIME and COMPOUND main effects (ANOVA), significant interaction term parameters (i.e., b_4 and b_5) indicated that the slope of firing rate as a function of time in the given treatment group significantly differs from the slope of firing rate after vehicle administration.

Results

D-Serine and DAAO Inhibition Reverses MK-801–Induced Cognitive Impairment in Passive Avoidance Test in Rats

Using the passive avoidance paradigm in rats, the likelihood ratio (LR) test of the Cox proportional hazard model revealed a significant global effect of MK-801 and D-serine on associative learning of rats (LR = 9.63, $P = .047$). The MK-801 group was used as reference (hazard ratio = 1). Rats treated with MK-801 showed impaired learning performance, and almost all of them entered the dark compartment with a mean latency of 119.6 ± 38.6 seconds (mean ± SEM) in contrast with the vehicle control group (latency: 248.4 ± 34.9 seconds), indicating a partial loss of the painful memory of the foot-shock (Fig. 1A). Figure 1C shows an inverse Kaplan-Meier plot of the cumulative step-through latencies of the groups. The model showed a significant decrease of hazard ratio of the control group (0.160, 95% CI = [0.034, 0.761], $P = .021$; Fig. 1E) for entering the dark zone, meaning that it was about 6 times more probable for MK-801–treated rats to enter the dark zone than control rats. D-serine effectively reversed the cognitive impairment of rats in the passive avoidance test at the 640-mg/kg dose: D-serine showed a decreased hazard ratio of 0.263 (95% CI = [0.069, 0.996], $P = .049$) for entering the dark zone (latency: 219.8 ± 41.0 seconds) in reference to MK-801, which indicates that D-serine attenuated the MK-801–induced inhibition of fear conditioning.

In another set of experiments, MK-801 again impaired the cognitive performance and all animals entered the dark compartment (latency: 32.2 ± 18.0 seconds; Fig. 1B). The vehicle group showed a significantly lower risk to enter the dark zone (hazard ratio: 0.058, 95% CI = [0.012, 0.280], Cox model: LR = 20.59, $P < .001$; Fig. 1D, F) with a latency of 253.2 ± 32.7 seconds. The 0.1-mg/kg dose of CPD30 was effective to improve cognitive impairment caused by MK-801: the hazard ratio in the MK-801 + 0.1-mg/kg CPD30 group was 0.373 (95% CI = [0.144, 0.968], $P = .043$) in reference to the MK-801 group, and the latency for entering the dark zone was 108.9 ± 36.4 seconds.

D-Serine and CPD30 Increase Spontaneous Firing Rate Following Local Application

The local effects of D-serine and CPD30 were studied in 25 animals, and D-serine and CPD30 were tested on 44 and 45 neurons, respectively. Figure 2A and C show parts of representative raw firing rate histograms from experiments with D-serine and CPD30, respectively. Both compounds increased the spontaneous firing rate of the neurons: increase in firing rate was found in significantly more neurons than decrease as a result of the application of D-serine or CPD30 (29 vs 6 and 31 vs 2 neurons, respectively, binomial test: $P < .001$ for both compounds; Fig. 2B, D). D-serine significantly increased the spontaneous firing rate from a control value of 2.83 ± 0.88 Hz to 5.96 ± 1.45 Hz (t = 2.285, df = 43, $P = .027$; Fig. 2E). During the iontophoresis of CPD30, the spontaneous firing rate increased from 3.42 ± 0.77 Hz to 4.88 ± 1.04 Hz (t = 3.295, df = 44, $P = .002$). No significant difference was found between the effects of D-serine and CPD30 according to contingency table analysis and chi-squared test ($\chi^2 = 2.484$, $P = .289$). The average normalized firing rate increase was 2.74 ± 0.20 and 2.45 ± 0.19 after the iontophoresis of D-serine and CPD30, respectively (t = 1.025, df = 50, $P = .310$).

Effects of Local Application of D-Serine and CPD30 on NMDA-Evoked Firing Responses

The majority of the recorded neurons responded to the application of D-serine and CPD30 with a marked increase of the NMDA-evoked firing rate (Fig. 2B, D). The increase in NMDA-evoked firing rate was significant during the administration of either D-serine ($P < .001$) or CPD30 ($P < .01$) according to the binomial test (22 vs 4, and 20 vs 5 neurons, respectively). During the local administration of D-serine, the prior control NMDA-evoked firing rate of 44.43 ± 3.60 Hz significantly increased to 53.10 ± 4.57 Hz (t = 3.277, $P = .002$), while CPD30 augmented the NMDA-evoked firing rate from 36.25 ± 3.37 Hz to 42.67 ± 4.46 Hz (t = 2.390, $P = .021$) (Fig. 2F). The distribution of effects on NMDA-evoked firing rate was similar after the iontophoresis of D-serine and CPD30 ($\chi^2 = 0.300$, $P = .860$). Application of D-serine resulted in an increase of NMDA-evoked firing responses (normalized change: 1.56 ± 0.07) that was not significantly different from the effect of CPD30 (1.75 ± 0.11; D-serine vs CPD30: t = –1.463, df = 40, $P = .151$).

Effects of D-Serine and CPD30 on the Spontaneous Firing Rate of Neurons Following Systemic Administration

Systemic administration of the compounds resulted in similar responses to their local application. Saline (0.9% NaCl) and a 1280-mg/kg dose of D-serine were tested in 13 and 15 recording sessions, respectively. Vehicle (PEG-400) and 0.1- and 1.0-mg/kg doses of CPD30 were tested in 13, 14, and 14 separate recording sessions, respectively. Figure 3 shows raw firing rate histograms from representative electrophysiological recordings. D-serine significantly increased the spontaneous firing rate over time (main effect: F[6, 58.8] = 5.377, $P < .001$) as the spontaneous firing rate was significantly higher than the pre-injection control value (T0: 0.68 ± 0.18 Hz) at 50 minutes after the administration of D-serine (T50: 3.45 ± 1.12 Hz; normalized firing rate: 2.78 ± 1.12) and further increased at T60 (4.32 ± 1.65; normalized firing rate: 3.64 ± 1.65) (Fig. 4A).

Similarly to D-serine, CPD30 significantly increased the spontaneous firing rate of the neurons in both doses (0.1 mg/kg: $P < .001$; 1.0 mg/kg: $P = .001$; Fig. 4C). However, the spontaneous firing rate also increased after vehicle administration, and there was no difference between the effect sizes of the vehicle and test compound treatments (TIME × COMPOUND interaction: $P = .992$).

Effects of D-Serine and CPD30 on NMDA-Evoked Neuronal Firing Following Systemic Administration

D-serine increased the NMDA-evoked firing rate (main effect: $P < .001$) in contrast to saline, which did not cause any significant change in the NMDA-evoked firing ($P = .715$) (Fig. 4B). The effect of D-serine developed within the first 20 minutes (T20: 37.0 ± 4.5 Hz vs T0: 26.0 ± 4.0; normalized firing rate at T20: 11.1 ± 4.5) and

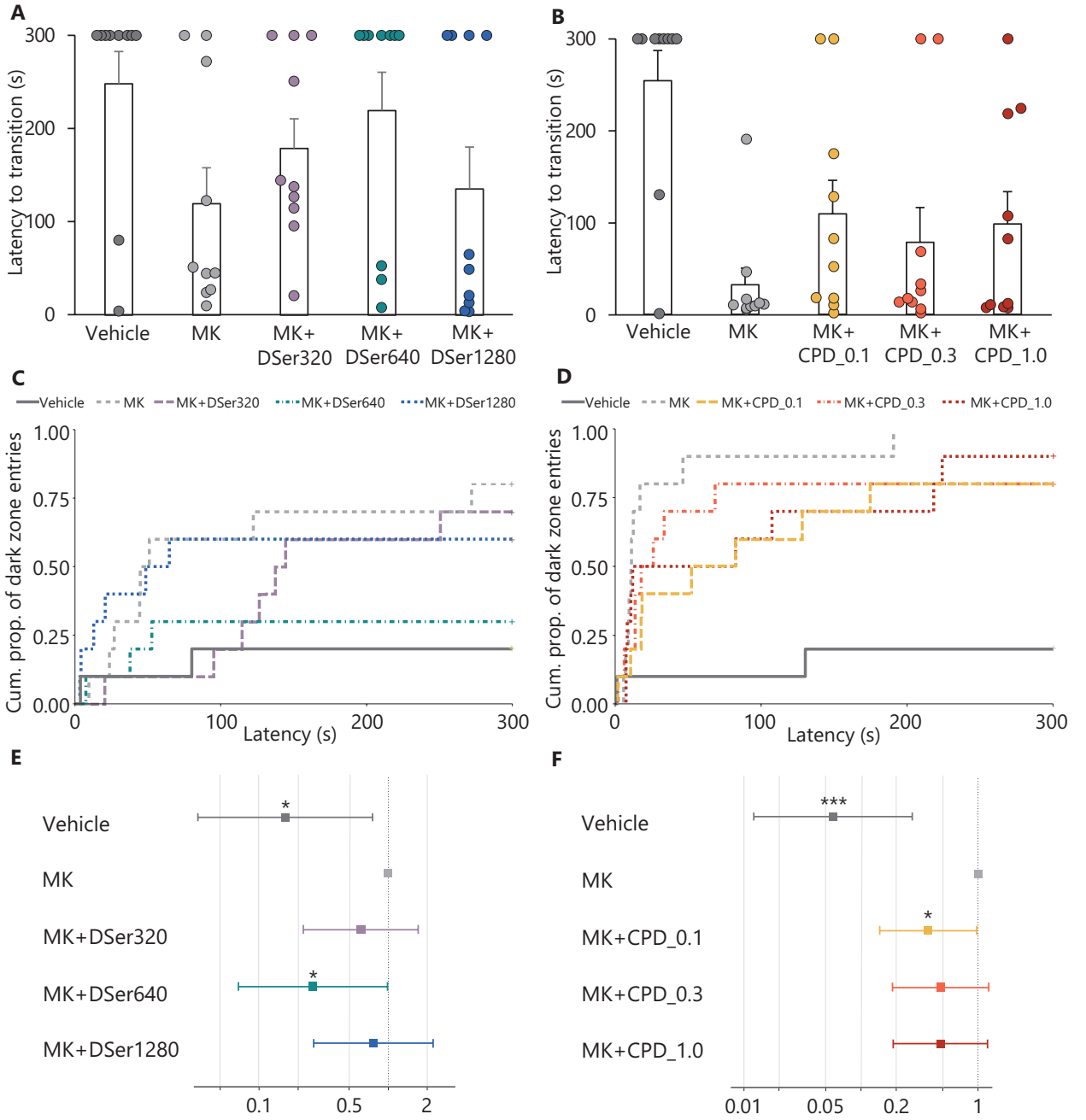

Figure 1. Effects of D-serine (A, C, E) and Compound 30 (B, D, F) on passive avoidance performance of Wistar rats. Performance was represented on bar charts with raw data points (A–B) and on cumulative events (i.e., transition to the dark zone) plots (C–D). Forest plots (E–F) represent hazard ratios (effect sizes) and significant effects of treatments calculated from Cox-proportional hazards model *$P<.05$, ***$P<.001$. CPD_0.1, Compound 30 0.1 mg/kg; CPD_0.3, Compound 30 0.3 mg/kg; CPD_1.0, Compound 30 1.0 mg/kg; Dser1280, D-serine 1280 mg/kg; DSer320, D-serine 320 mg/kg; Dser640, D-serine 640 mg/kg; MK, MK-801.

lasted until the end of the sessions. D-serine reached the maximum effect at 50 minutes after administration (T50: 45.9±7.5). In a separate set of experiments, CPD30 also increased the NMDA-evoked firing rate at both doses (0.1 mg/kg: $P=.001$; 1.0 mg/kg: $P<.001$), while vehicle did not affect NMDA-evoked firing ($P=.988$; Fig. 4D). The change in the NMDA-evoked firing rate was significant from 30 minutes after the administration of 0.1 mg/kg CPD30 (T30: 43.6±3.3 Hz vs T0: 37.0±4.2 Hz, normalized firing rate at T30: 6.6±3.3; $P<.05$). The highest effect was reached at 50 minutes after the injection (T50: 47.1±3.6; normalized firing rate: 10.1±3.6; $P<.01$). CPD30 was more potent in the 1.0-mg/kg dose because it was effective already at 10 minutes after administration (T10: 38.8±4.4 vs T0: 33.1±3.5, normalized firing rate at T10: 5.7±4.4; $P<.05$), and the firing responses to NMDA further changed converging to a plateau (maximum effect at T50: 45.2±4.5 Hz; normalized firing rate: 12.2±4.5; $P<.001$). The slopes of the effect of CPD30 at both doses were significantly higher than those of the vehicle, indicating a significant effect of CPD30 over its vehicle: TIME×COMPOUND ($F[2, 193.6]=4.727$, $P=.010$); parameter estimates (TIME×[CPD_0.1]: 0.12±0.06, $df=193.7$, $t=2.036$, $P=.043$; TIME×[CPD_1.0]: 0.18±0.06, $df=193.1$, $t=3.027$, $P=.003$).

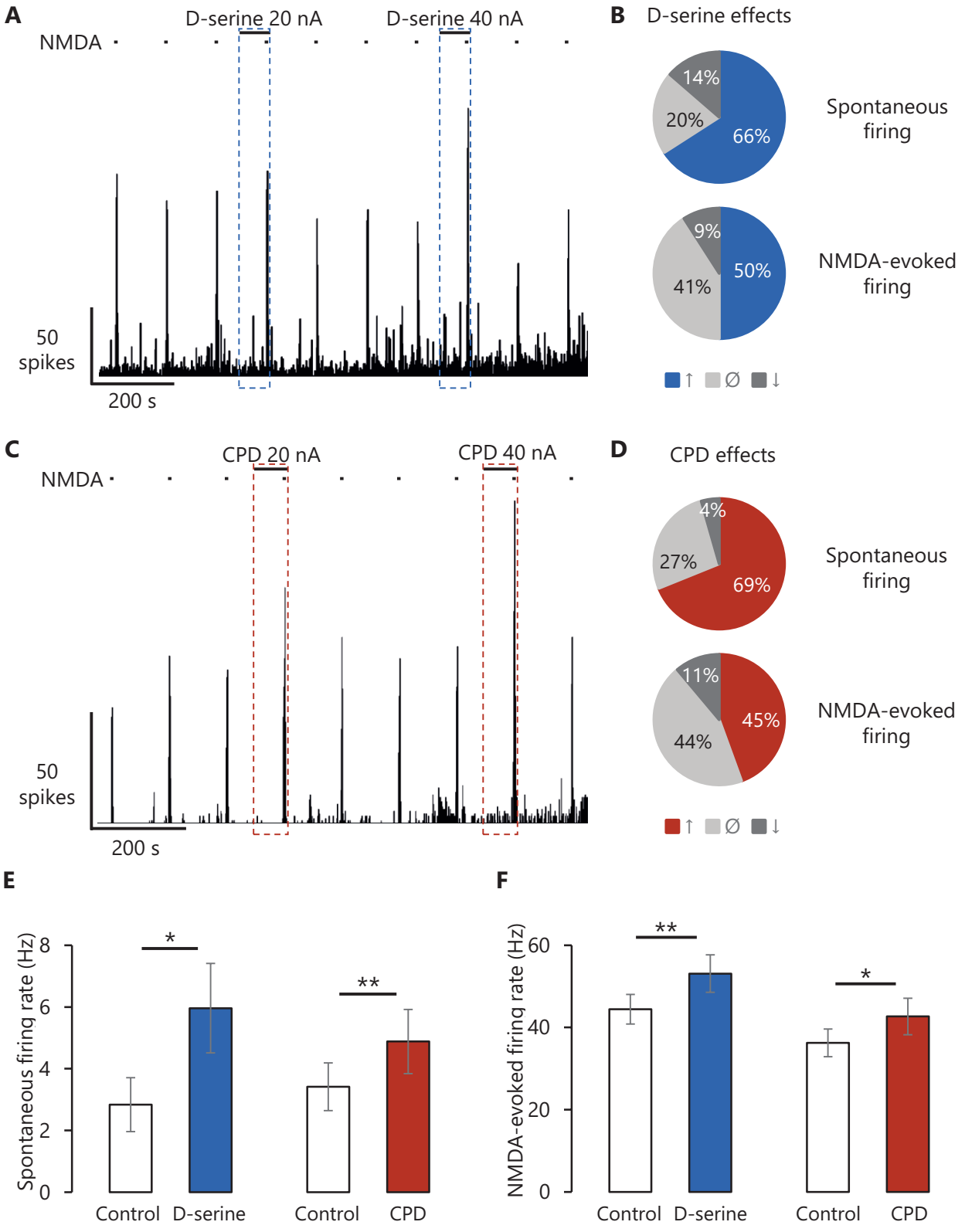

Figure 2. Effects of locally administered D-serine and Compound 30 (CPD) on the spontaneous and N-methyl-D-aspartate (NMDA)-evoked firing activity of neurons recorded in the hippocampal CA1 area. Firing rate histograms of representative recordings (A, C), distribution (right) of firing rate increasing (↑), decreasing (↓), and neutral (Ø) effects (B, D) of D-serine (A–B) and CPD (C–D) on the spontaneous and NMDA-evoked firing activity of neurons. Effect of D-serine and CPD on the mean spontaneous (E) and NMDA-evoked (F) firing rates of the tested neurons (sample sizes for D-serine and CPD were 44 and 45 neurons, respectively). Asterisks symbols above bars indicate that the given treatment resulted in averaged firing rate significantly higher than the control, *$P < .05$, **$P < .01$.

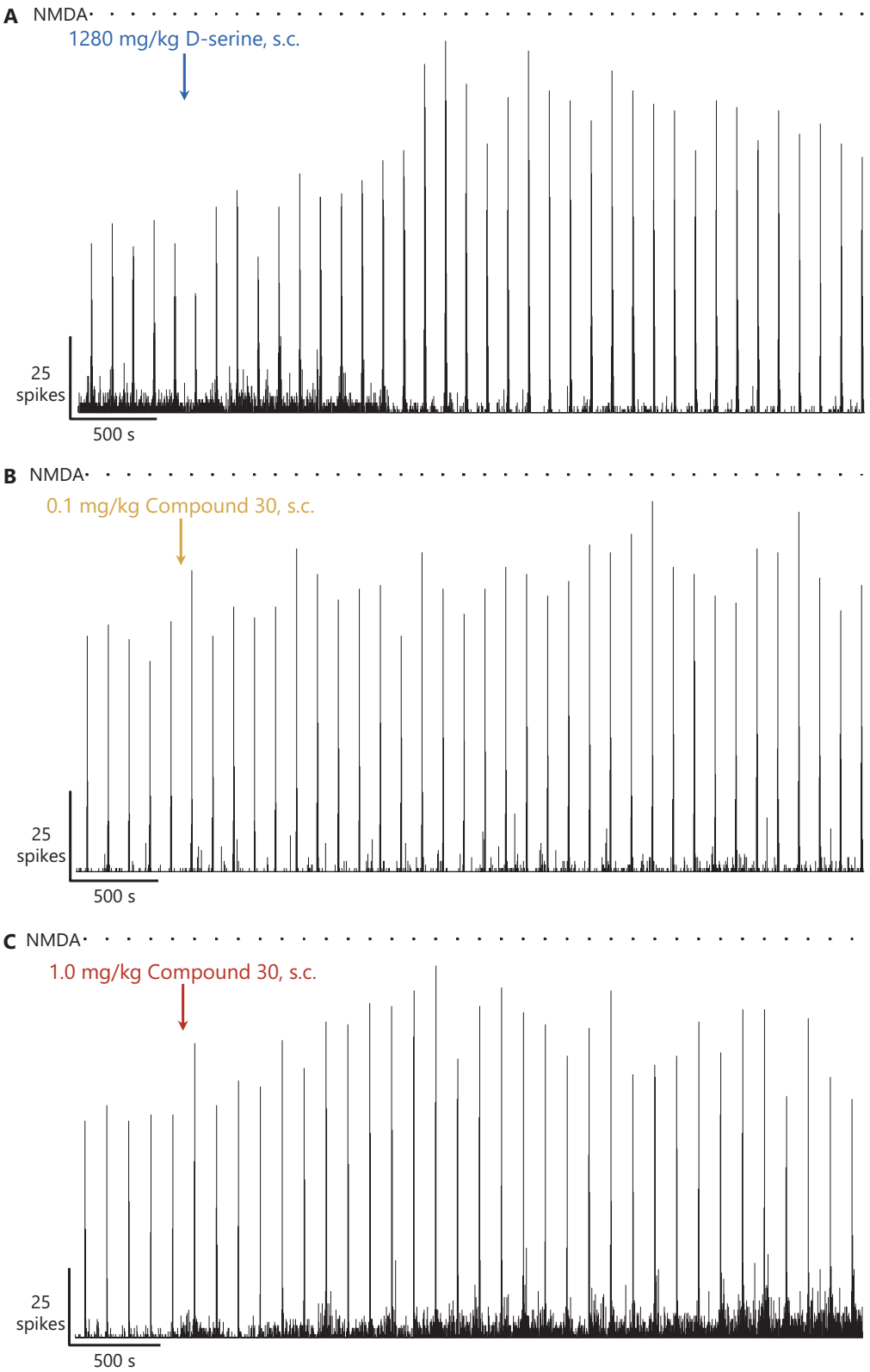

Figure 3. Effects of D-serine and Compound 30 (CPD30) on neuronal firing after systemic administration: representative electrophysiological recordings from CA1 hippocampal neurons. Each vertical line represents the number of firing events in a given 1 second time window. Markers above the firing rate histogram indicate repetitive iontophoretic deliveries of N-methyl-D-aspartate. The arrow shows the time when D-serine (A) and different doses of CPD30 (B–C) were subcutaneously (s.c.) injected.

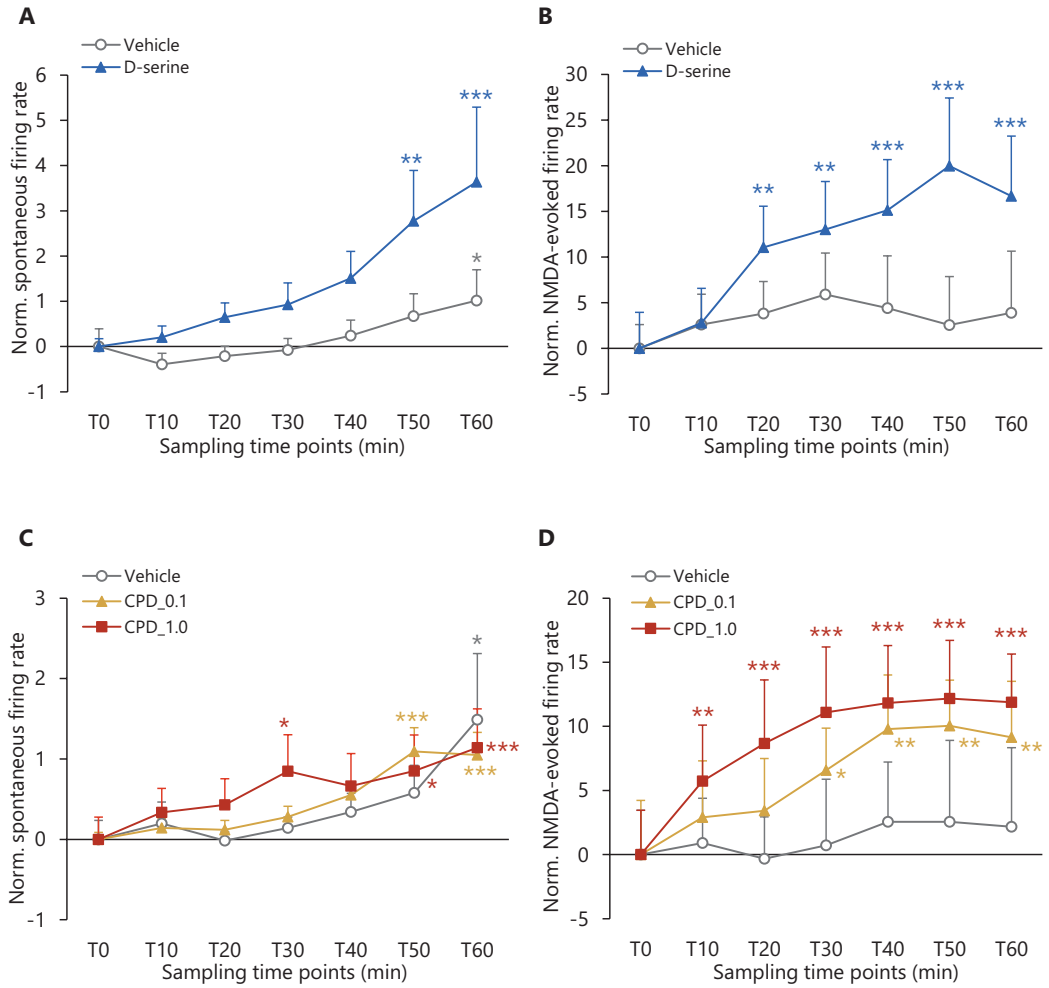

Figure 4. Effects of D-serine and Compound 30 (CPD30) on neuronal firing after systemic administration: mean ± SEM plots showing the changes of normalized spontaneous (A, C) and N-methyl-D-aspartate–evoked (B, D) firing rates after the injection of D-serine (A–B), CPD30 (C–D), and their vehicles. D-serine was applied at a 1280-mg/kg dose (4 mL/kg volume), and CPD30 was applied at 0.1-mg/kg (CPD_0.1) and 1.0-mg/kg (CPD_1.0) doses (5 mL/kg volume). T0, control firing rate before the administration of the test compound or vehicle.; T10-T60, firing rate at 10–60 minutes after the injection. Data were normalized using the mean pre-injection control firing rate values. Hypothesis testing was conducted on the normalized variable, and post-hoc comparisons were corrected using Holm's method. *$P < .05$, * $P < .01$, ***$P < .001$ in a given post-injection time point (T10–T60) show significant difference from the corresponding baseline control (T0).

Discussion

The aim of the present study was to investigate the positive neurocognitive effects of D-serine and DAAO inhibitor CPD30, 2 compounds that may have possible enhancing effects on NMDARs in the cerebral cortex. The investigations showed converging evidence on functional enhancement in 2 system-level domains: (1) cognitive performance was improved in a simple reference memory task, and (2) firing activity and cellular responsiveness of neurons in the hippocampus were increased as the result of elevated extracellular levels of D-serine.

The cognitive effects of the 2 compounds were investigated in the passive avoidance task, which is a fear-aggravated test used to evaluate learning and simple reference memory in rodent models of CNS disorders, especially those associated with psychiatric and neurocognitive conditions. The passive avoidance task is frequently used in conjunction with pharmacologically induced amnesia and learning impairment. In the present experiment, memory impairment induced by MK-801 was significantly reversed by 640 mg/kg D-serine or 0.1 mg/kg CPD30. These results suggest that the increase of D-serine levels either by D-serine supplement treatment or by DAAO enzyme inhibition results in a cognitive enhancing effect.

There are several studies that showed the reversal of adverse behavioral effects of MK-801 using pharmacological agents acting directly on the allosteric site of the NMDAR or on endogenous D-serine levels (Karasawa et al., 2008; Shimazaki et al., 2010; Hondo et al., 2013). However, the mechanism of the reversal of MK-801 effect has not yet been uncovered. It was shown that the glycine sites of NMDARs are not saturated in vivo, and earlier data also suggest that MK-801 in low doses does not bind to all available NMDARs in the brain (Price et al., 1988; Fernandes et al., 2015). Therefore, we find it possible that the reversal of MK-801 induced cognitive deficits reported both in the previous studies and in our current paper was achieved through the potentiation of NMDARs that were not blocked by MK-801. Another possible explanation is that D-serine acts on presynaptic receptors and increases the release of glutamate, which in turn activates postsynaptic receptors (Li and Han, 2007; Ohi et al., 2015).

Some earlier studies also suggest that decreased DAAO activity can be accompanied by memory-enhancing effects. Maekawa et al. (2005) reported an increased performance of DAO–/– mice in the Morris water maze test, while enhanced hippocampal long-term potentiation was also observed in the mutant mice. In another study, Pritchett et al. (2015) confirmed that DAO–/– mice showed better memory performance than their wild-type littermates in spatial and object recognition tests as well as in odor discrimination and spontaneous alternation tasks. However, mutant mice also showed increased anxiety in a set of behavioral tests, in line with the observations of other research groups (Labrie et al., 2009a). In addition, a natural mutant line of mice that carries a single point mutation (G181R) resulting in the complete lack of DAAO activity also showed better memory performance. These mutant mice performed better than their wild-type counterparts, especially in the reversal or extinction phases of behavioral tests, suggesting more flexible responses and better adaptation to changing environmental conditions (Labrie et al., 2009b). Furthermore, genetic lack of DAAO activity also reverses behavioral abnormalities in a genetic model of schizophrenia (Labrie et al., 2010). In summary, earlier results indicating the improvement of memory performance in mice genetically lacking DAAO enzyme are now confirmed by our behavioral pharmacological results using the DAAO inhibitor agent CPD30.

In concert with the behavioral results, the present electrophysiological data showed for the first time, to our knowledge, that both locally administered D-serine and DAAO inhibitor CPD30 significantly modulated spontaneous and NMDA-evoked neuronal activity in the rat hippocampus under in vivo conditions. Both test compounds exerted a significant increase in the firing rate of most neurons, and there was no significant difference between the effects of the 2 test compounds in the case of local application. Thus, indirectly increasing the extracellular D-serine level with the DAAO inhibitor had similar efficacy as the direct application of D-serine itself. Additionally, D-serine and CPD30 significantly increased both the spontaneous and the NMDA-evoked firing rate of hippocampal neurons also after their systemic administration.

The enhancement of NMDAR function by D-serine is in line with previously reported results. For example, Salt (1989) reported that D-serine increased NMDA-evoked firing responses of thalamic neurons by approximately 66% (i.e., to 166% of the control) during the parallel microiontophoretic administration of the 2 compounds. In contrast, in a whole-cell recording experiment, Martina et al. (2003) showed that D-serine acts only on a minority of pyramidal cells under in vitro conditions (5 of 22 neurons), which may also be explained by the fact that collateral inhibition also plays a crucial role in the balance of the activity of local neuronal circuits. These findings are in line with our present results as D-serine had no effect in almost one-half of the neurons tested, which may be a beneficial response of the local circuits against excessive stimulation of the receptors and a defense against excitotoxicity. In contrast to the action of D-serine on the cellular levels, only a limited information is available on the effects of DAAO inhibition on NMDAR function in vivo. Intraoral administration of the DAAO inhibitor 4H-furo[3,2-b] pyrrole-5-carboxylic acid (SUN) 3–4 hours prior to testing significantly increased NMDA-mediated long-term potentiation in hippocampus of anaesthetized rats, and the same treatment with SUN exerted a pronounced procognitive effect on recognition memory (Hopkins et al., 2013). In another study, SUN also enhanced the hippocampal theta rhythm, which is a known electrophysiological correlate of memory consolidation and retrieval and is mainly generated by the hippocampus and adjacent temporal lobe structures (Strick et al., 2011). In line with the above pieces of evidence, here we confirmed that local administration of CPD30 to the vicinity of hippocampal pyramidal neurons rapidly increased both spontaneous and NMDA-evoked neuronal firing. The results of the present experiments provide novel evidence on the in vivo, cellular level electrophysiological correlates of D-serine supplementation and of DAAO inhibition in the hippocampus under in vivo conditions.

The present findings in the electrophysiological experiments are highly coherent with the behavioral results, as the systemic application of CPD30 at as low as 0.1 mg/kg significantly increased the NMDAR-mediated hippocampal activity within 1 hour and consequently improved cognitive performance in the passive avoidance task in this relevant time window. Our results, therefore, suggest that the rapid increase of hippocampal NMDAR-mediated activity by DAAO inhibition may be a plausible mechanism underlying the observed procognitive effects of CPD30. However, we have to note that reversal of MK-801–induced blockade of NMDARs was not tested in this set of experiments. Further electrophysiological measurements are needed to provide direct pharmacological evidence for restoration of decreased NMDA function by D-serine and DAAO inhibitors.

Additionally, our present observations after the systemic (i.p.) administration of D-serine and CPD30 showed important differences between the consequences of direct D-serine supplementation and DAAO inhibition. We found that the DAAO inhibitor compound CPD30 exerted a much more pronounced effect on the NMDA-evoked firing activity compared with D-serine. Overall, we can conclude that D-serine and CPD30 exerted almost similar effect when they were locally applied; however, when systemic administration was used, the DAAO inhibitor compound CPD30 had a more notable effect both on spontaneous and NMDA-evoked firing activity. The background of this observation can be the insufficient brain penetration of D-serine. Due to this, the very high doses of D-serine that may be required for optimal efficacy (Hashimoto and Chiba, 2004) may already cause side effects and raise safety concerns. Thus, using effectively high doses of D-serine for NMDA receptor modulation might lead to excitotoxicity and can cause several serious peripheral side effects, such as nephrotoxicity (Ganote et al., 1974). Considering the translational drug developmental approach, the indirect facilitation of the receptor function by the elevation of D-serine level by the inhibition of DAAO enzyme-mediated degradation (Verrall et al., 2010) may be more effective and less harmful strategies for facilitating NMDAR function.

In conclusion, both D-serine and the DAAO inhibitor were active in modulating cognitive performance in the passive avoidance paradigm in rats. Furthermore, both compounds significantly increased the firing activity and responsiveness of hippocampal neurons, which might be a potential cellular-level phenomenon behind the observed behavioral effects. These experiments are dedicated to providing novel comparative evidence and result in deeper understanding of how DAAO inhibition and elevated brain D-serine levels enhance cognitive function. Results may further facilitate the development of novel therapeutic avenues and medical applications of DAAO inhibition in neurological and psychiatric disorders.

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Interest Statement

CPD30 was synthesized at Orion Pharma, Finland, and supplied for Gedeon Richter Plc. in the framework of a research collaboration. Neither honoraria nor payments were received for authorship. Authors G.K., P.P., B.F., B.L., and G.L. are employed by Gedeon Richter Plc. This does not alter our adherence to journal policies on sharing data and materials. The remaining authors (L.V.N., Z.K.B., and I.H.) are academic researchers and declare that the research was conducted in the absence of any commercial, non-financial, or financial relationships that could be construed as a potential conflict of interest.

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