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Pro-cognitive effects of dual tacrine derivatives acting as cholinesterase inhibitors and NMDA receptor antagonists

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ABSTRACT

Therapeutic options for Alzheimer's disease are limited. Dual compounds targeting two pathways concurrently may enable enhanced effect. The study focuses on tacrine derivatives inhibiting acetylcholinesterase (AChE) and simultaneously N-methyl-D-aspartate (NMDA) receptors. Compounds with balanced inhibitory potencies for the target proteins (K1578 and K1599) or increased potency for AChE (K1592 and K1594) were studied to identify the most promising pro-cognitive compound. Their effects were studied in cholinergic (scopolamine-induced) and glutamatergic (MK-801-induced) rat models of cognitive deficits in the Morris water maze. Moreover, the impacts on locomotion in the open field and AChE activity in relevant brain structures were investigated. The effect of the most promising compound on NMDA receptors was explored by *in vitro* electrophysiology. The cholinergic antagonist scopolamine induced a deficit in memory acquisition, however, it was unaffected by the compounds, and a deficit in reversal learning that was alleviated by K1578 and K1599. K1578 and K1599 significantly inhibited AChE in the striatum, potentially explaining the behavioral observations. The glutamatergic antagonist dizocilpine (MK-801) induced a deficit in memory acquisition, which was alleviated by K1599. K1599 also mitigated the MK-801-induced hyperlocomotion in the open field. *In vitro* patch-clamp corroborated the K1599-associated NMDA receptor inhibitory effect. K1599 emerged as the most promising compound, demonstrating pro-cognitive efficacy in both models, consistent with intended dual effect. We conclude that tacrine has the potential for development of derivatives with dual *in vivo* effects. Our findings contributed to the elucidation of the structural and functional properties of tacrine derivatives associated with optimal *in vivo* pro-cognitive efficacy.

1. Introduction

Alzheimer's disease (AD), a prevalent neurodegenerative disorder causing dementia [1], poses a growing socioeconomic challenge due to population aging and limited therapeutic success [2]. This multifactorial disease involves complex pathophysiology [2], including cholinergic neuron loss and, apparently, N-methyl-D-aspartate (NMDA) glutamate receptor overstimulation [3–6]. However, the pathophysiological

Abbreviations: AChE, acetylcholinesterase; AD, Alzheimer's disease; ANOVA, analysis of variance; BChE, butyrylcholinesterase; DMSO, dimethyl sulfoxide; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; IC_{50}, the half maximal inhibitory concentration; K1578, 7-chloro-1*H*,2*H*,3*H*-cyclopenta[*b*]quinolin-9-amine hydrochloride; K1592, 1-chloro-6*H*,7*H*,8*H*,9*H*,10*H*-cyclohepta[*b*]quinolin-11-amine hydrochloride; K1594, 6-methyl-1,2,3,4-tetrahydroacridin-9-amine hydrochloride; K1599, 7-methoxy-1*H*,2*H*,3*H*-cyclopenta[*b*]quinolin-9-amine hydrochloride; MK-801, dizocilpine; MWM, Morris water maze; NMDA receptor, N-methyl-D-aspartate receptor; SEM, standard error of the mean; VEH, vehicle-treated group; 7-MEOTA, 7-methoxytacrine.

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processes are widely interconnected [6–9] and together drive disease progression.

Current therapy offers only palliative effects via acetylcholinesterase (AChE) inhibitors, indirectly stimulating the cholinergic system, and the NMDA receptor antagonist memantine, assumed to mitigate NMDA receptor overactivation [10]. From other experimental approaches, single-target strategies have not succeeded, [2] except for recently presented immunotherapies that are questionable [11]. Given the disease's complexity, a polypharmacology approach targeting multiple pathways concurrently emerges as a more promising strategy [12,13]. This strategy can enhance therapeutic efficacy while minimizing side effects [10,12,14,15]. Polypharmacology employs combination therapy or multi-target drugs, enabling interaction with multiple (usually two) molecular targets simultaneously [12–14]. A plethora of such dual-target molecules, often combining AChE inhibition with another relevant mechanism of action, have been synthesized and subjected to preclinical testing [16–18].

A favorable combination of mechanisms of action is AChE inhibition and NMDA receptor inhibition [10,13,14]. Glutamatergic and cholinergic pathologies in AD intricately interact [10], are present in the same phase of disease progression [14], and drugs targeting them show complementary actions [10,19–21], making simultaneous targeting of these pathways a highly rational approach that could possibly increase effectiveness [10,13,14]. Co-administration of AChE inhibitors and NMDA receptor antagonists enhanced cognitive recovery in rodent models [22,23] as well as in patients [15,24], leading to drug approval (Namzaric) [25]. This combination therapy sets the stage for the development of multi-target drugs, which are preferred as they can overcome some limitations of combination therapy [12–14].

Tacrine (Fig. 1**a**), the first AChE inhibitor for AD, displayed therapeutic potential [26] but was discontinued due to hepatotoxicity risk [27]. However, efforts to develop safer derivatives continue [28,29]. Importantly, tacrine was described to inhibit NMDA receptors as well [30–32]. Tacrine hence represents an interesting parent molecule for the development of dual-target compounds with low molecular weight, overcoming the problems associated with high molecular weight and resulting suboptimal drug-likeness of many multi-target drugs, particularly those designed by the linking approach [14]. However, tacrine displays unbalanced affinities between NMDA receptors and cholinesterases, with a high preference towards the latter [30–32]. This issue is crucial. If we want to take advantage of both effects, the affinity to both targets should be balanced. More recently, we have shown that a less toxic tacrine derivative, 7-methoxytacrine (7-MEOTA; Fig. 1**b**), exhibited higher efficacy towards NMDA receptors than the parent tacrine, and promising *in vivo* effects [32]. However, 7-MEOTA's affinity towards NMDA receptors and cholinesterases was still unbalanced. We set forth to investigate the biological profile of other tacrine derivatives [33,34], out of which four compounds were highlighted, endowed with balanced inhibitory potencies for target proteins (K1578 and K1599) or with increased inhibitory potency for AChE (K1592 and K1594 (Fig. 1**c–f**)) [33]. These compounds were meticulously investigated in the current extensive *in vivo* efficacy study, emphasizing dual-target effects and cognition restoration. This research builds on our previous study by Gorecki et al. [33].

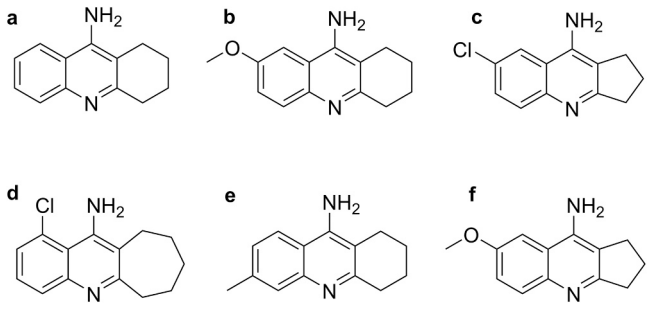

Fig. 1. Chemical structures of tacrine (**a**) and its derivatives created by introducing substituents on the aromatic core and/or altering the size of the cycloalkyl moiety attached to the aromatic region: 7-MEOTA (**b**), K1578 (7-chloro-1*H*,2*H*,3*H*-cyclopenta[*b*]quinolin-9-amine; **c**), K1592 (1-chloro-6*H*,7*H*,8*H*,9*H*,10*H*-cyclohepta[*b*]quinolin-11-amine; **d**), K1594 (6-methyl-1,2,3,4-tetrahydroacridin-9-amine; **e**), and K1599 (7-methoxy-1*H*,2*H*,3*H*-cyclopenta[*b*]quinolin-9-amine; **f**). Compounds in this study were used in the form of hydrochloride salts.

Generally, a balanced target affinity, specifically AChE and NMDA receptor inhibition, is a prerequisite for multi-target directed ligands [14]. On the other hand, tuning the most appropriate combination of affinities may be essential [14], and even a tiny difference in affinity or subtype selectivity can result in a completely different phenotype *in vivo*. The selected compounds showed various combinations of affinities towards cholinesterases (AChE and butyrylcholinesterase, BChE) and different NMDA receptor subtypes (GluN1/GluN2A vs. GluN1/GluN2B, known to play different roles in pathological states [35,36]) [33]. K1578 and K1599 demonstrated rather balanced inhibitory potencies towards NMDA receptors and cholinesterases, with IC_{50} values in the one- to two-digit micromolar range. The IC_{50} values of K1578 followed the order AChE < BChE < GluN1/GluN2B < GluN1/GluN2A, while those of K1599 displayed the GluN1/GluN2A < AChE < BChE < GluN1/GluN2B relationship. K1592 and K1594 were also micromolar NMDA receptor inhibitors but with strong, submicromolar inhibitory potency for AChE, even surpassing that of tacrine. K1592 was also a submicromolar inhibitor of BChE. Regarding NMDA receptors, K1592 and K1594 showed a slight preference for GluN1/GluN2A and GluN1/GluN2B, respectively. (For exact IC_{50} values and structure-activity relationship see [33].) Of note, all the selected compounds are able to cross the blood-brain barrier and are available in the brain *in vivo* [33].

The main aim of the current study was to investigate and compare the pro-cognitive effects of the compounds, given by their cholinergic and glutamatergic characteristics, in rat models of cognitive deficits based on cholinergic and glutamatergic dysfunction. We used scopolamine and dizocilpine (MK-801) to induce these deficits, as they are well-established and widely used cognition impairers in rodent models [37–39]. Scopolamine acts as a competitive antagonist of muscarinic acetylcholine receptors [40], which are crucial for memory processes [41,42]. This model partly replicates the cholinergic deficit seen in AD patients [4]. On the other hand, MK-801 is a potent selective antagonist of NMDA receptors [43,44], which play a significant role in learning and memory [45,46], and MK-801 is used to selectively impair cognition in animal models [37,39]. Our experiments assessed spatial memory and cognitive flexibility, both relevant to AD [47,48], and included additional analyses such as open field behavior, *in vitro* patch-clamp study focusing on NMDA receptors, and AChE inhibition in specific brain regions to understand the compounds' mechanisms of action. The pro-cognitive, behavioral, AChE- and NMDA receptor-inhibitory effects of the parent compound tacrine in these assays are well-documented in the literature [32,38,49,50]. Finally, we identified the most beneficial tacrine derivative that was effective in both models.

2. Materials and methods

2.1. Animals

Adult male Wistar rats (*Rattus norvegicus;* 280–460 g, 2–3 months) obtained from the Velaz breeding facility (Czech Republic) were used. They were housed in pairs in transparent plastic boxes (23 × 38 × 23 cm) in an animal room at the National Institute of Mental Health, Czech Republic, with constant temperature (22.5 °C), humidity, and 12:12 h light/dark cycle, with free access to water and food pellets. Experiments were performed during the light phase of the day following a one-week acclimatization period. The experiments were conducted in accordance with the guidelines of European Union directive 2010/63/

EU and Act No. 246/1992 Coll. on the protection of animals against cruelty, and were approved by the Animal Care and Use Committee of the National Institute of Mental Health (reference number MZDR 51755/2018–4/OVZ). In determining the sample size, we considered relevant literature on corresponding methodologies [51–53] as well as the principles of Replacement, Reduction, and Refinement of animals used in research.

2.2. Drugs and administration to animals

Compounds K1578, K1592, K1594, and K1599 (in the form of hydrochloride salts) synthesized according to [33] were used (HPLC purity > 97%). Other compounds for animal administration were purchased from Sigma-Aldrich (St. Louis, MO, USA). Doses are expressed as the salt form of the drugs.

2.2.1. Administration of compounds

Compounds K1578, K1592, K1594, and K1599 were administered at 1 mg/kg by dissolving them in 5% dimethyl sulfoxide (DMSO) in physiological saline, resulting in a 1 mg/mL concentration, with an injection volume of 1 mL/kg. For the 5 mg/kg dose, the compounds were dissolved in the same vehicle at a 2 mg/mL concentration and administered at 2.5 mL/kg. MK-801 ((+)-MK-801 hydrogen maleate; 0.2 mg/kg for the open field test, 0.1 mg/kg for Morris water maze; MWM) and scopolamine hydrobromide (2 mg/kg) were dissolved in physiological saline at 1 mL/kg. All compounds were administered intraperitoneally (ip), and the route and doses were chosen based on previous experience [33,37,39,54]. Co-administration of the 5 mg/kg dose of K1592 with MK-801 was not performed due to side effects.

2.2.2. Timing of administration

In the behavioral experiments, MK-801 was administered 30 min before, scopolamine 20 min before, and K1578, K1592, K1594, and K1599 15 min before the start of the behavioral testing, based on their pharmacokinetic properties [33]. In the AChE enzyme activity assay, the drugs were administered 30 min before euthanizing the subjects, which reflects the timing during the MWM testing.

2.3. Behavioral experiments

2.3.1. Morris water maze

2.3.1.1. Treatment groups. The rats were pseudo-randomly assigned to one of the 18 treatment groups listed in Table 1 (upper part). Each group received two injections: one containing the study compound and another containing either MK-801 or scopolamine, as indicated by the group name. The vehicle group (VEH) received the DMSO vehicle (2.5 mL/kg) and saline. The "scopolamine" and "MK-801" groups received scopolamine or MK-801, respectively, along with the DMSO vehicle (2.5 mL/kg).

2.3.1.2. Morris water maze apparatus. The MWM is a routinely used test of spatial cognition for rodents [55]. The apparatus consisted of a 180 cm diameter gray plastic pool with a hidden platform (circular, 10 cm diameter, transparent, submerged 1 cm below the water surface). The water (40 cm depth) was maintained at 23°C and tinted with non-toxic gray dye. No visual cues were present on the maze walls, requiring rats to rely on constant room-based cues for navigation.

2.3.1.3. Testing procedure. The experiment spanned 5 consecutive days. The drugs were administered daily before testing. On days 1–4, the position of the hidden platform remained constant in the center of a selected quadrant for acquisition trials. On day 5, the hidden platform was moved to the center of the opposite quadrant for reversal trials [55,56]. Reversal learning reflects cognitive flexibility, a complex process enabling adjustment of subject's behavior to changes in the environment (switching the search strategy to the new platform position) [55,57,58]. Each day, rats underwent 8 swims from different starting positions at the perimeter of the pool (labeled N, S, E, W, NE, NW, SE, SW, in a pseudo-random order) with an intertrial interval of 4–7 min. Rats were placed into the pool facing its inner wall and given 60 s to locate the platform. If a rat failed to find the platform, it was gently guided to it by the experimenter. The rat remained on the platform for 15 s to acquire spatial cues before being removed.

Table 1
Treatment groups and *n* in behavioral experiments.

Groups	*n*	Groups	*n*
Morris water maze			
Scopolamine model		**MK-801 model**	
VEH (shared for both models)	7		
Scopolamine	7	MK - 801	6
K1578 (1 mg/kg) + scopolamine	6	K1578 (1 mg/kg) + MK-801	6
K1578 (5 mg/kg) + scopolamine	6	K1578 (5 mg/kg) + MK-801	6
K1592 (1 mg/kg) + scopolamine	6	K1592 (1 mg/kg) + MK-801	6
K1592 (5 mg/kg) + scopolamine	6		
K1594 (1 mg/kg) + scopolamine	6	K1594 (1 mg/kg) + MK-801	5
K1594 (5 mg/kg) + scopolamine	6	K1594 (5 mg/kg) + MK-801	6
K1599 (1 mg/kg) + scopolamine	9	K1599 (1 mg/kg) + MK-801	6
K1599 (5 mg/kg) + scopolamine	9	K1599 (5 mg/kg) + MK-801	6
Open field			
Intact phenotype		**MK-801 phenotype**	
VEH (1 mL/kg)	8	VEH (1 mL/kg) + MK-801	6
VEH (2.5 mL/kg)	6	VEH (2.5 mL/kg) + MK-801	7
K1578 (1 mg/kg)	6	K1578 (1 mg/kg) + MK-801	6
K1578 (5 mg/kg)	6	K1578 (5 mg/kg) + MK-801	8
K1592 (1 mg/kg)	6	K1592 (1 mg/kg) + MK-801	6
K1594 (1 mg/kg)	6	K1594 (1 mg/kg) + MK-801	6
K1594 (5 mg/kg)	6	K1594 (5 mg/kg) + MK-801	6
K1599 (1 mg/kg)	6	K1599 (1 mg/kg) + MK-801	6
K1599 (5 mg/kg)	6	K1599 (5 mg/kg) + MK-801	6

Note: *n:* number of subjects per group.

2.3.1.4. Data collection. Rat positions were monitored using a camera mounted above the pool, connected to tracking software (EthoVision 11, Noldus, Netherlands). The dependent variable reflecting cognitive performance was escape latency (the latency to find the platform [55]), represented as the daily mean value. In cases where a rat did not find the platform, a latency of 60 s was recorded.

2.3.1.5. Control procedure. Although the Morris water maze is relatively robust against motivational differences potentially emerging as side-effects of pharmacological interventions [55], the mean swimming speed of the animals from day 1–5 was analyzed to control for potential motor or motivational impairment [55] (ANOVA with Tukey's multiple comparisons test, scopolamine model and MK-801 model analyzed separately). Neither scopolamine nor MK-801 decreased the speed (data not shown), nor did we observe any evident motor impairment in the rats during MWM testing, suggesting low risk of confounding MWM results by sensorimotor deficits.

2.3.2. Open field test

2.3.2.1. Treatment groups. The rats were pseudo-randomly assigned to one of the 18 treatment groups listed in Table 1 (lower part). Animals received either 1 (intact phenotype groups) or 2 (MK-801 phenotype groups) injections, as indicated by their group names. (Due to side effects, co-administration of MK-801 with the 5 mg/kg dose of K1592 was not conducted.) The "VEH" groups received the specified volumes of the DMSO vehicle. The "VEH + MK-801" groups received the DMSO vehicle along with MK-801. Two different volumes of the DMSO vehicle were used in the control animals to provide appropriate control groups for the 1 and 5 mg/kg treatment groups. However, as there was not statistically

significant difference between the distance moved by the "VEH 1 mL/kg" and "VEH 2.5 mL/kg" groups, as well as between "VEH 1 mL/kg + MK-801" and "VEH 2.5 mL/kg + MK-801" groups (t-test), these groups were merged and referred to as "VEH" and "VEH + MK-801", respectively.

2.3.2.2. Data source clarification. Data from the intact phenotype groups (i.e., without MK-801 co-administration) have been previously published for side effect assessment in [33]. These experiments were not replicated in the current publication to minimize the use of laboratory animals.

2.3.2.3. Open field test apparatus and procedure. The open field test used a black plastic square arena (80 × 80 cm), located in a controlled-light room. Rats were placed in the center of the arena, and their activity was tracked for 10 min using a camera connected to tracking software (EthoVision 14, Noldus, Netherlands). The arena was thoroughly cleaned after each animal. The dependent variable was the distance moved by the animal.

2.4. Biochemistry experiments - In situ acetylcholinesterase activity assay

2.4.1. Sacrifice and tissue preparation

Separate sets of experimentally naïve rats were administered the compounds at 1 mg/kg via the same route and at a corresponding time point as the rats subjected to the MWM task. The rats were pseudo-randomly assigned to the groups listed in Table 2. The VEH groups, receiving the DMSO vehicle in volumes of 1 mL/kg or 2.5 mL/kg, were merged into one control group as no statistically significant alterations of AChE activity within the analyzed brain regions were observed between the groups (data not shown).

All rats were euthanized via decapitation in accordance with ethical protocols. Thirty minutes after drug administration, the decapitation procedure was conducted humanely, and the brains were swiftly excised and rinsed with ice-cold saline. The brain was then divided sagittally into two halves. The prefrontal cortex, striatum, and hippocampus, structures involved in the studied cognitive functions [58–60], were dissected from the left half of the brain, while the entire right half of the brain (including the right half of the cerebellum) was left intact and referred to as whole brain homogenate.

The collected tissue samples were weighed, immediately frozen at dry ice, and stored at –80 °C. The samples were homogenized on ice using a solubilization solution composed of 10 mM TRIS, 1 M NaCl, 50 mM $MgCl_2$, 1% Triton X-100, and a cocktail of protease (cOmplete®, Roche) and phosphatase (PhosSTOP®, Roche) inhibitors (pH = 7.2). This homogenization process employed an IKA® ULTRA-TURRAX® with dispersing elements S10N-5G and 8G, resulting in 10% (w/v) tissue homogenates. Right upon collecting the homogenate, a small portion of the homogenate was used to determine the protein concentration using the Bradford dye (Merck) binding assay [61]. The remaining homogenate was appropriately labeled, frozen, and stored for subsequent assessment of AChE enzyme activity.

Table 2
Treatment groups and *n* in biochemical experiments - AChE activity assay.

Groups	*n*
VEH (1 mL/kg)	6
VEH (2.5 mL/kg)	8
K1578 (1 mg/kg)	10
K1592 (1 mg/kg)	6
K1594 (1 mg/kg)	6
K1599 (1 mg/kg)	10

Note: *n:* number of subjects per group.

2.4.2. Acetylcholinesterase enzyme activity assessment

Despite the concurrent BChE-inhibiting effects of the compounds, we focused solely on AChE activity, as BChE expression in the key structures, the hippocampus and striatum, is marginal or absent [62,63]. The enzymatic activity of AChE was assessed using the spectrophotometric Ellman's method, a widely recognized approach for quantifying cholinesterase activity [64]. This method relies on the reaction between acetylthiocholine iodide and 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), generating a yellow product comprising mercapto-2-nitrobenzoic acid and its dissociated forms. This chemical transformation occurs under controlled conditions at pH 8.0, with the highest absorption coefficient observed at 412 nm, having a specific value of 13.6×10^{-3} M^{-1} cm^{-1}.

Briefly, thawed sample aliquots were significantly diluted (10–15 times) with a solubilization solution, resulting in a final volume of 100 µL per sample. These diluted samples were then dispensed in triplicate into a 96-well microplate. To provide a basis for comparison, two control groups were included: one contained 100 µL of the solubilization solution (the negative control), and the other contained 100 µL of diluted AChE enzyme sourced from Electrophorus electricus (Merck) as the positive control. Following this, 50 µL of a 1.25 mM DTNB solution, prepared in 0.1 M Phosphate buffer at pH 8.0, was added to each well. Subsequently, the samples were incubated for 5 min at room temperature. Next, the microplate was transferred to a microplate reader, specifically the Infinite M200Pro by Tekan®. The enzymatic reaction was initiated by introducing 50 µL of an acetylthiocholine iodide solution, prepared at a concentration of 1.75 mM in 0.1 M Phosphate buffer with a pH of 8.0. The reaction kinetics, involving the increase in absorbance at $\lambda = 412$ nm, were monitored and recorded over a period of 10 min, with readings taken at 1-min intervals. Subsequently, the specific activity of the samples was computed as activity units per milligram of protein present in each well. Finally, the enzyme activities within the tacrine derivative groups were expressed relative to the control group, quantified as fold changes in comparison to the control group.

2.5. In vitro electrophysiology experiments

Whole-cell patch-clamp measurements were performed on transfected HEK293 cells expressing rat version of the GluN1–4a/GluN2A (GluN1/GluN2A) receptors using an Axopatch 200B amplifier (Molecular Devices) as described [32,65]. The cells were placed in an extracellular solution (ECS) containing (in mM): 160 NaCl, 2.5 KCl, 10 HEPES, 10 D-glucose, 0.2 EDTA, and 0.7 $CaCl_2$ (pH 7.3 with NaOH). Glass patching pipettes (3–6 MΩ resistance) were prepared using a P-1000 puller (Sutter Instruments) and filled with intracellular solution (ICS) containing (in mM): 125 gluconic acid, 15 CsCl, 5 EGTA, 10 HEPES, 3 $MgCl_2$, 0.5 $CaCl_2$, and 2 ATP-Mg salt (pH 7.2 with CsOH). Electrophysiological recordings were conducted at room temperature using membrane potentials from -80–60 mV (with a step of 20 mV); the junction potential was not subtracted. The GluN1/GluN2A receptor currents were induced by fast application of 1 mM glutamate in the continuous presence of 100 µM glycine. The stock solution of K1599 was freshly dissolved in DMSO and diluted to a final working concentration of 30 µM. All chemicals described above were obtained from Merck. Data were analyzed using Clampfit 10.2 software (Molecular Devices). The relative inhibition of glutamate-induced GluN1/GluN2A receptor currents by K1599 was calculated by dividing the current value during compound inhibition by the value of the steady-state current after channel opening, and then expressing this number as a percentage.

2.6. Statistics

Data from individual experiments were examined separately due to variations in the protocols employed. Specifically, data from the MWM reversal and AChE activity assay were subjected to one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparisons test when appropriate. For the open field test, a two-way ANOVA was

employed with factors being tacrine derivative treatment and phenotype (i.e., MK-801), followed by Dunnett's multiple comparisons test to investigate treatment effects (comparison vs. VEH group of the corresponding phenotype). Aforementioned data underwent rigorous scrutiny, involving checks for outliers using the robust regression and outlier removal (ROUT) method with a Q value set at 1%, and assessments for normality (Shapiro-Wilk test, Kolmogorov-Smirnov test) and for homogeneity of variances (Brown-Forsythe test). In cases where the assumption of homogeneity of variances was not met, Welch's ANOVA was employed, followed by corrections for multiple comparisons using Dunnett's T3 multiple comparisons test.

Mean daily latencies from MWM acquisition were analyzed using two-way repeated measures ANOVA, with the day as the repeated factor, followed by Dunnett's multiple comparisons test to investigate the main treatment effect by comparing mean latencies across the acquisition period. Separate ANOVAs were performed for each compound in the behavioral experiments. The predetermined level of significance was established at $p \leq 0.05$. All statistical analyses were executed using GraphPad Prism 8 software (San Diego, USA).

3. Results

3.1. Morris water maze

3.1.1. Scopolamine-induced model of cognitive deficit

3.1.1.1. Acquisition. Two-way repeated measures ANOVAs of the escape latencies on the MWM acquisition days were conducted separately for the K1578, K1592, K1594, and K1599 groups, along with their respective control groups. These analyses revealed significant effects of treatment ($F(3, 22) = 8.737$, $p = 0.0005$; $F(3, 22) = 8.801$, $p = 0.0005$; $F(3, 22) = 6.026$, $p = 0.0037$; and $F(3, 28) = 5.424$, $p = 0.0045$,

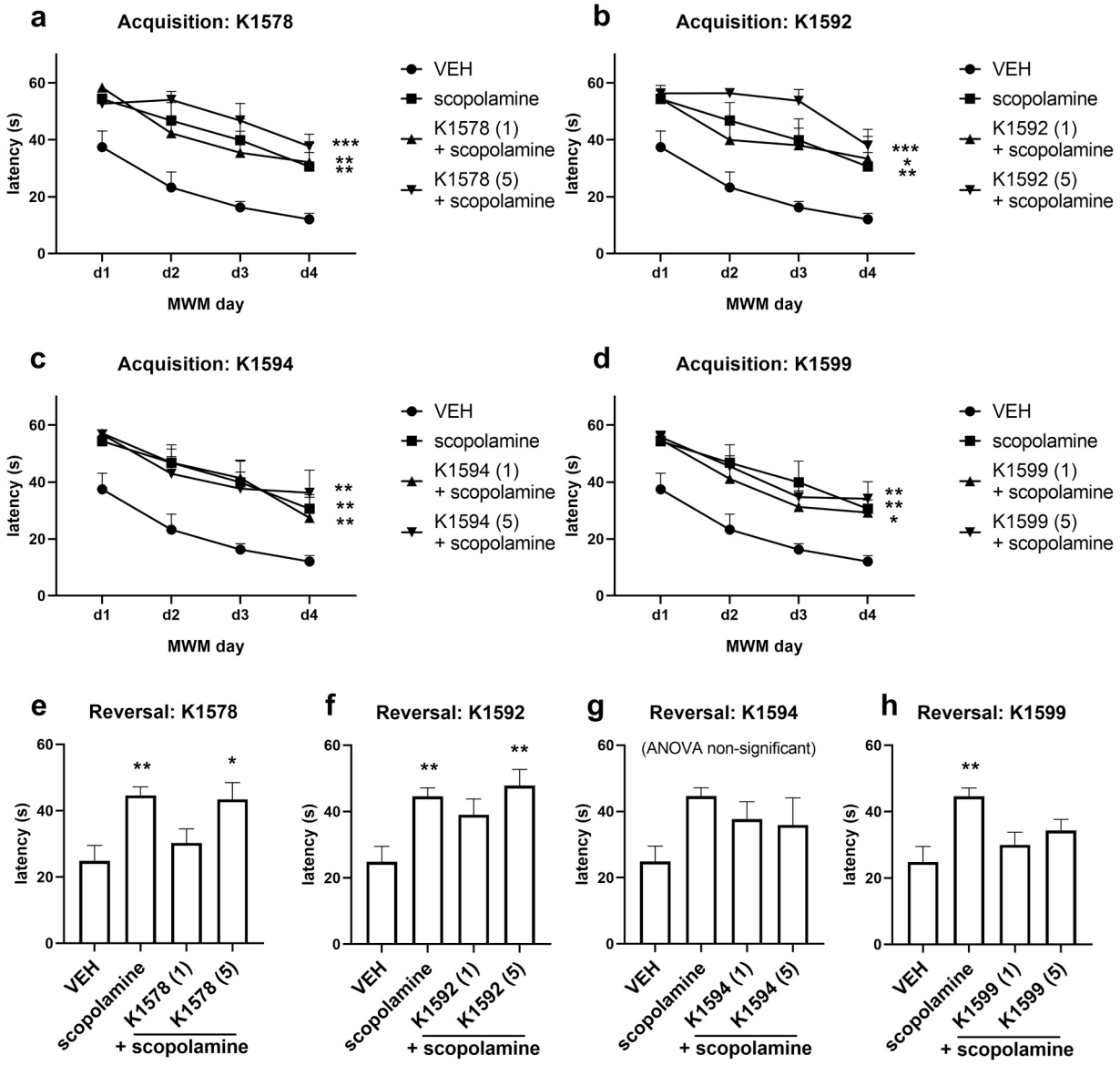

Fig. 2. Morris water maze: scopolamine-induced model of cognitive deficit in the acquisition and reversal phases. The graphs show the effects of K1578 (**a**), K1592 (**b**), K1594 (**c**), and K1599 (**d**) on escape latency during the acquisition phase, where none of the compounds ameliorated the deficit of spatial learning. The remaining graphs display the effects of K1578 (**e**), K1592 (**f**), K1594 (**g**), and K1599 (**h**) in the reversal phase, where K1578 (1 mg/kg) and K1599 (at both doses), and marginally K1592 (1 mg/kg; see in the text), mitigated the scopolamine-induced deficit of reversal learning. VEH – vehicle, the numbers in brackets denote the dose applied (mg/kg). Data are presented as the mean + SEM, * vs. VEH, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 6$–9 animals per group. Statistical significance was determined using two-way repeated measures ANOVA (a–d) or ANOVA (e, f, h) followed by Dunnett's multiple comparisons tests.

respectively) and testing day ($F(3, 66) = 29.18$; $F(3, 66) = 30.19$; $F(3, 66) = 38.86$; and $F(3, 84) = 48.91$, respectively; $p < 0.0001$ for all). We observed decreasing escape latencies along with an increase of testing day as the rats learned. No significant interactions between treatment and day factors were observed.

Post hoc tests focused on the main treatment effect, which involved examining mean group latencies over all the acquisition days. These tests revealed a significantly increased mean latency in the scopolamine group compared to the VEH group ($p < 0.01$), indicating a deficit in spatial learning.

The groups co-administered with scopolamine and K1578 ($p = 0.0045$ for 1 mg/kg dose and $p = 0.0004$ for 5 mg/kg; Fig. 2**a**), K1592 ($p = 0.0102$ for 1 mg/kg and $p = 0.0002$ for 5 mg/kg; Fig. 2**b**), K1594 ($p = 0.0074$ for 1 mg/kg and $p = 0.0068$ for 5 mg/kg; Fig. 2**c**), or K1599 ($p = 0.0163$ for 1 mg/kg and $p = 0.0037$ for 5 mg/kg; Fig. 2**d**) displayed increased latencies compared to VEH. This indicates that all the compounds failed to reduce the scopolamine-induced deficit of spatial learning.

3.1.1.2. Reversal. On the 5th day, the platform was moved to the opposite quadrant to study reversal learning. ANOVAs of the mean escape latencies of the K1578 groups ($F(3, 22) = 5.650$, $p = 0.0050$), K1592 groups ($F(3, 22) = 5.989$, $p = 0.0038$), and K1599 groups ($F(3, 28) = 4.631$, $p = 0.0094$) revealed a treatment effect. The post hoc tests showed that scopolamine increased the escape latency ($p < 0.01$ vs. VEH), indicating a deficit in reversal learning.

Regarding the K1578 groups, the post hoc test showed that the latency of the K1578 (1 mg/kg) + scopolamine group did not significantly differ from VEH ($p = 0.6901$). It suggests mitigation of the scopolamine-induced cognitive deficit by K1578. However, animals treated with the higher dose of K1578 (5 mg/kg) showed increased latencies ($p = 0.0130$ vs. VEH), indicating that this dose of K1578 did not mitigate the cognitive deficit (Fig. 2**e**).

Similarly, the post hoc test focused on the K1592 groups revealed that the latency of the K1592-treated animals (1 mg/kg) did not differ from VEH, suggesting a possible mitigation of the cognitive deficit. However, the reduction of the cognitive deficit was only minor, as seen from the group means (Fig. 2**f**). Considering the 5 mg/kg dose of K1592, this group again showed increased latency ($p = 0.0025$ vs. VEH), indicating no mitigation of the cognitive deficit (Fig. 2**f**).

In K1599 groups, the post hoc test showed that the latency of the group K1599 (1 mg/kg) + scopolamine as well as K1599 (5 mg/kg) + scopolamine did not differ from VEH ($p = 0.6329$ and 0.1881, respectively), suggesting alleviation of the cognitive deficit by both doses of K1599 (Fig. 2**h**).

In the case of K1594 groups, ANOVA found no differences between the groups; therefore, the post hoc test could not be performed (Fig. 2**g**).

To sum up, K1578 (1 mg/kg) and K1599 (both doses), and marginally also K1592 (1 mg/kg), mitigated the scopolamine-induced deficit of reversal learning.

3.1.2. MK-801-induced model of cognitive deficit

3.1.2.1. Acquisition. Separate two-way repeated measures ANOVAs of the escape latencies of the K1578, K1592, K1594, and K1599 groups revealed the effect of treatment ($F(3, 21) = 8.155$, $p = 0.0009$; $F(2, 16) = 9.957$, $p = 0.0016$; $F(3, 20) = 6.459$, $p = 0.0031$; and $F(3, 21) = 5.263$, $p = 0.0073$, respectively) and testing day ($F(3, 63) = 31.07$; $F(3, 48) = 36.15$; $F(3, 60) = 26.17$; and $F(3, 63) = 40.36$, respectively; $p < 0.0001$ for all), with no interaction between these factors. The post hoc tests focused on the main treatment effect showed an increased mean latency of the MK-801 group ($p < 0.01$ vs. VEH), confirming a deficit in spatial learning.

The groups co-administered with MK-801 and K1578 ($p = 0.0013$ for 1 mg/kg dose and $p = 0.0039$ for 5 mg/kg; Fig. 3**a**), K1592 (1 mg/kg; $p = 0.0047$; Fig. 3**b**), or K1594 ($p = 0.0033$ for 1 mg/kg and $p = 0.0161$ for 5 mg/kg; Fig. 3**c**), respectively, displayed significantly increased latencies compared to VEH as well. It indicates that these compounds

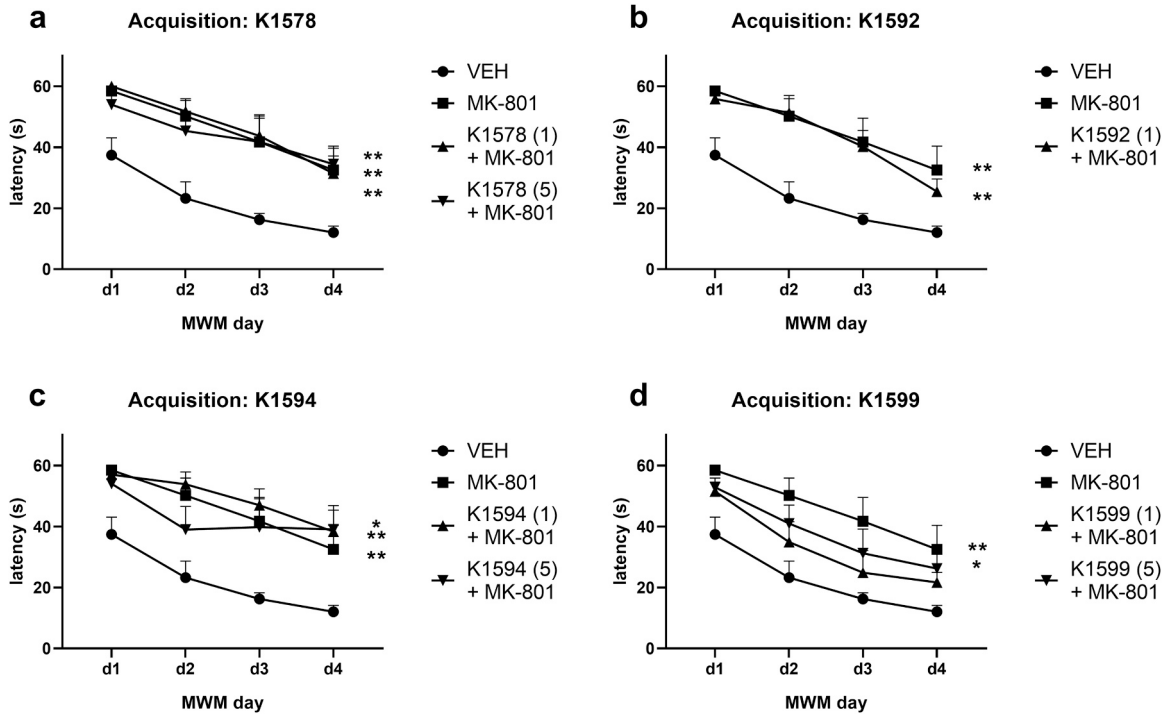

Fig. 3. Morris water maze: MK-801-induced model of cognitive deficit in the acquisition phase. The graphs illustrate the effects of the compounds K1578 (**a**), K1592 (**b**), K1594 (**c**), and K1599 (**d**) on escape latency. Only K1599 (1 mg/kg) ameliorated the MK-801-induced deficit of spatial learning. VEH – vehicle, the numbers in brackets denote the dose (mg/kg). Data are presented as the mean + SEM, * vs. VEH, * $p < 0.05$, ** $p < 0.01$. $n = 5$–7 animals per group. Statistical significance was determined using two-way repeated measures ANOVA followed by Dunnett's multiple comparisons tests.

did not mitigate the MK-801-induced cognitive deficit.

However, animals co-treated with MK-801 and K1599 at the 1 mg/kg dose did not exhibit a significant difference in latency compared to VEH ($p = 0.2017$), suggesting an improvement of the MK-801-induced cognitive deficit by K1599 at this lower dose. Notably, at the 5 mg/kg dose of K1599, the animals showed increased latency ($p = 0.0475$ vs. VEH), indicating a lack of ameliorative effect of K1599 at this dose (Fig. 3**d**).

In summary, only K1599 at the 1 mg/kg dose demonstrated an ameliorative effect on the MK-801-induced deficit of spatial learning.

3.1.2.2. Reversal. In the reversal phase of the experiment, MK-801 failed to induce a statistically significant cognitive deficit. Therefore, it was not possible to study the effects of the drugs in this part of the experiment (data not shown). ANOVAs of the latencies of the K1578, K1592, K1594, and K1599 groups failed to show significant differences among the means.

3.2. Open field test

To further investigate NMDA receptor-mediated actions, the effects of the compounds on locomotor activity were tested in two rat phenotypes: intact and MK-801-treated rats, where MK-801 induced hyperlocomotion.

Analysis of the distance moved by K1578 groups revealed the effect of K1578 ($F (2, 46) = 75.11$, $p < 0.0001$), MK-801 ($F (1, 46) = 144.8$, $p < 0.0001$), and the interaction of these factors ($F (2, 46) = 5.111$, $p = 0.0099$). K1578 (1 mg/kg) did not affect locomotion in intact animals but further increased hyperlocomotion in MK-801-treated animals ($p = 0.0165$ vs. VEH+MK-801). However, at 5 mg/kg, K1578 decreased locomotion in both intact and MK-801-treated rats ($p < 0.0001$ for both; Fig. 4**a**).

Analysis of the K1592 groups revealed the effect of K1592 and of MK-801 ($F (1, 34) = 40.19$ and $F (1, 34) = 67.16$, respectively, $p < 0.0001$ for both). K1592 (1 mg/kg) decreased locomotion in intact animals ($p = 0.0049$) and MK-801-treated animals ($p < 0.0001$; Fig. 4**b**). (Co-administration of the 5 mg/kg dose of K1592 with MK-801 was not performed due to side effects.)

Regarding K1594, the analysis revealed the effect of K1594 and MK-801 ($F (2, 44) = 15.54$ and $F (1, 44) = 68.95$, respectively, $p < 0.0001$ for both). At the dose of 5 mg/kg, K1594 decreased locomotion in both intact and MK-801-treated rats ($p = 0.0074$ and $p < 0.0001$, respectively), while at the lower dose, it did not affect locomotion in either group (Fig. 4**c**).

Finally, in the K1599 groups, the analysis showed the effect of K1599 ($F (2, 44) = 4.663$, $p = 0.0146$) and MK-801 ($F (1, 44) = 115.4$, $p < 0.0001$). At the dose of 5 mg/kg, K1599 mitigated the MK-801-induced hyperlocomotion ($p = 0.0044$ vs. VEH + MK-801), without affecting locomotion in intact animals. At the lower dose, it had no effect on locomotion in any group (Fig. 4**d**).

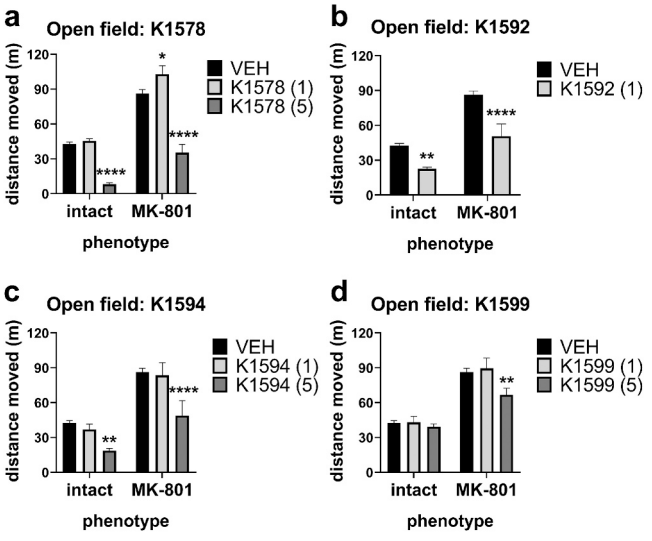

Fig. 4. Open field test. The graphs illustrate the effects of K1578 (**a**), K1592 (**b**), K1594 (**c**), and K1599 (**d**) on the distance moved by intact and MK-801-treated animals. VEH – vehicle, the numbers in brackets denote the dose (mg/kg). Data are presented as the mean + SEM, * vs. VEH group of the corresponding phenotype, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. $n = 6–14$ animals per group. A significant effect of both factors (treatment and phenotype) was determined using two-way ANOVA, followed by Dunnett's multiple comparisons tests.

3.3. In situ acetylcholinesterase activity assay

K1578, K1592, K1594, and K1599 are AChE inhibitors with IC_{50} values of 1.58, 0.223, 0.072, and 8.22 μM, respectively, as determined by a previous *in vitro* assay using the modified Ellman's method and human recombinant AChE [33]. To explore potential mechanisms underlying the results obtained in the scopolamine-induced animal model, the effects of these compounds (1 mg/kg ip) on AChE activity in selected rat brain structures were assessed using Ellman's method.

Spectrophotometric analyses of AChE activity did not reveal

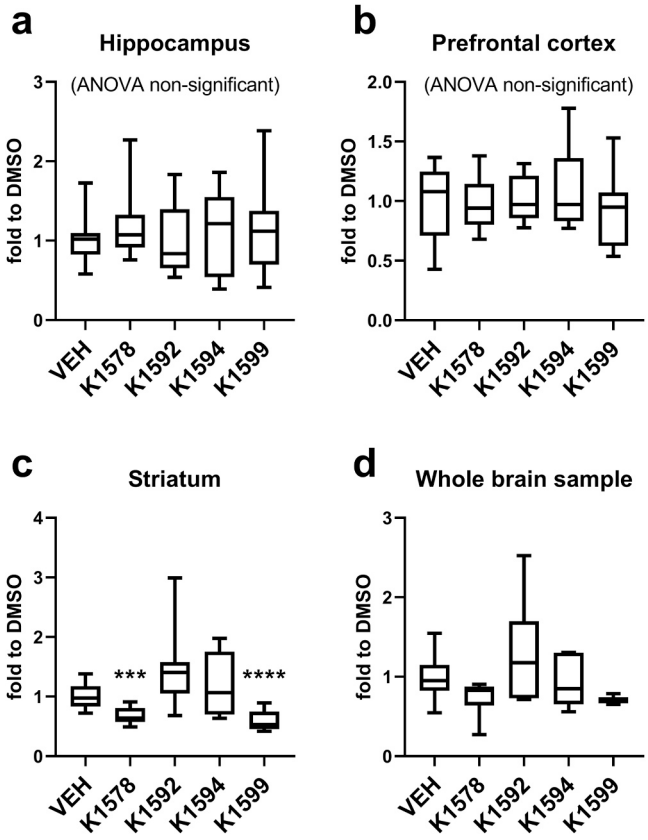

Fig. 5. Acetylcholinesterase activity. The graphs depict the effect of the compounds (1 mg/kg ip) on AChE activity in the hippocampus (**a**), prefrontal cortex (**b**), striatum (**c**), and whole brain sample (**d**). K1578 and K1599 decreased AChE activity in the striatum. VEH – vehicle. Data are presented as the median with minimum to maximum range, * vs. VEH, *** $p < 0.001$, **** $p < 0.0001$. VEH samples AChE enzyme activities reached the following absolute values (a) 15.19 ± 3.09 U/mg protein, (b) 9.810 ± 1.54 U/mg protein, (c) 26.05 ± 3.27 U/mg protein, and (d) 27.38 ± 3.36 U/mg protein. Significance was determined by ANOVA (graphs c, d), followed by Dunnett's multiple comparisons tests.

significant alterations in either the hippocampus (Fig. 5**a**) or the prefrontal cortex (Fig. 5**b**) following treatment with the compounds. ANOVA did not show a significant treatment effect.

In stark contrast, the analyses unveiled a substantial reduction in AChE activity in the striatum ($W(4.000, 18.85) = 12.77$, $p < 0.0001$). Specifically, K1578 led to a 32% decrease in AChE activity ($p = 0.0003$), while K1599 induced a 42% decrease ($p < 0.0001$). The results hence suggest that the selected doses of these compounds induce brain region-specific changes in AChE activity. Conversely, K1592 and K1594 did not produce statistically significant changes in AChE enzyme activity in the striatum compared to the control group (Fig. 5**c**).

The analysis of AChE activity in the whole brain sample revealed a treatment effect ($F(4, 38) = 3.741$, $p = 0.0116$). However, the Dunnett's multiple comparisons test did not detect any significant differences between the groups (Fig. 5**d**).

3.4. In vitro electrophysiology

K1578, K1592, K1594, and K1599 act as NMDA receptor inhibitors. They exhibit IC_{50} values of 21.01, 7.29, 17.05, and 4.16 μM, respectively, for GluN1/GluN2A receptors, and 8.69, 22.07, 7.83, and 14.56 μM, respectively, for GluN1/GluN2B receptors at a membrane potential of –60 mV [33]. As only K1599 ameliorated the MK-801-induced pathologies in the current study, we conducted a detailed *in vitro* electrophysiology study focusing on its interaction with GluN1/GluN2A receptors to investigate its beneficial effects further.

Whole-cell patch-clamp measurements were performed on transfected HEK293 cells expressing the rat version of GluN1–4a/GluN2A (GluN1/GluN2A). Relative inhibition of glutamate-induced GluN1/GluN2A receptor currents by K1599 was measured at different membrane potentials. Our findings indicate that K1599 inhibits GluN1/GluN2A receptors (Fig. 6**a**) across all studied membrane potentials. However, the inhibitory effect was more pronounced at negative membrane potentials (Fig. 6**b**).

4. Discussion

The present study revealed diverse *in vivo* effects of acute pretreatment with tacrine derivatives, which act as dual inhibitors of cholinesterases and NMDA receptors [33], in cholinergic and glutamatergic animal models of cognitive deficit. This research extends the findings of our earlier study led by Gorecki et al. [33].

4.1. Cholinergic effects

The first part of the study focused on the interactions of the compounds with the cholinergic system, as acetylcholine modulates learning and memory processes [41,66]. Consistent with the literature, scopolamine induced cognitive deficits in both spatial learning in acquisition trials [38,53,67,68] and reversal learning [68,69] in the MWM. It is known that these deficits can be mitigated by indirect stimulation of the cholinergic system by various AChE inhibitors including tacrine [38,53,67,68]. In the current study, K1578 and K1599 alleviated the reversal learning deficits, with K1599 being active over a wider dose range (both tested doses). On the other hand, none of the compounds were effective against the deficit of spatial learning in acquisition trials.

To understand these effects, we conducted an *in situ* AChE inhibition study using Ellman's method in separate sets of animals administered with the compounds at 1 mg/kg via the same route and timing as the rats subjected to MWM. We primarily focused on the hippocampus, which is important for spatial learning during MWM acquisition trials [59,70], and the striatum, which mediates spatial reversal learning and cognitive flexibility in general [58,71,72]. In particular, the intact cholinergic system in these structures seems crucial, as proven by the disrupting effect of locally (intrahippocampally or intrastriatally) infused scopolamine on spatial discrimination learning [73] and reversal learning [74], respectively.

In the hippocampus, none of the compounds significantly inhibited AChE, possibly explaining the lack of impact on spatial learning. It is unclear whether the compounds are fully ineffective or if different doses than those used in our study would be necessary to restore spatial memory. The known inverted U-shaped dose-response relationship and narrow therapeutic window of many AChE inhibitors [38,75–77] raise the possibility that the latter may be true. Similarly as in the hippocampus, AChE activity in the prefrontal cortex remained unchanged.

However, K1578 and K1599 significantly inhibited AChE (by 32% and 42%, respectively) in the striatum. The same dose of these compounds also mitigated the scopolamine-induced deficit of reversal learning in the rats subjected to the MWM. There is strong evidence that the cholinergic system in the striatum plays a key role in reversal learning, but not in the initial acquisition phase [58,72,74]. Specifically, cholinergic interneurons in the dorsomedial striatum are assumed to be crucial for establishing new strategies when conditions change [58]. Therefore, we suggest that there could be a causal relationship between the inhibition of striatal AChE by K1578 and K1599 and their positive effects on reversal learning.

Our results suggest that the different effects on acquisition versus

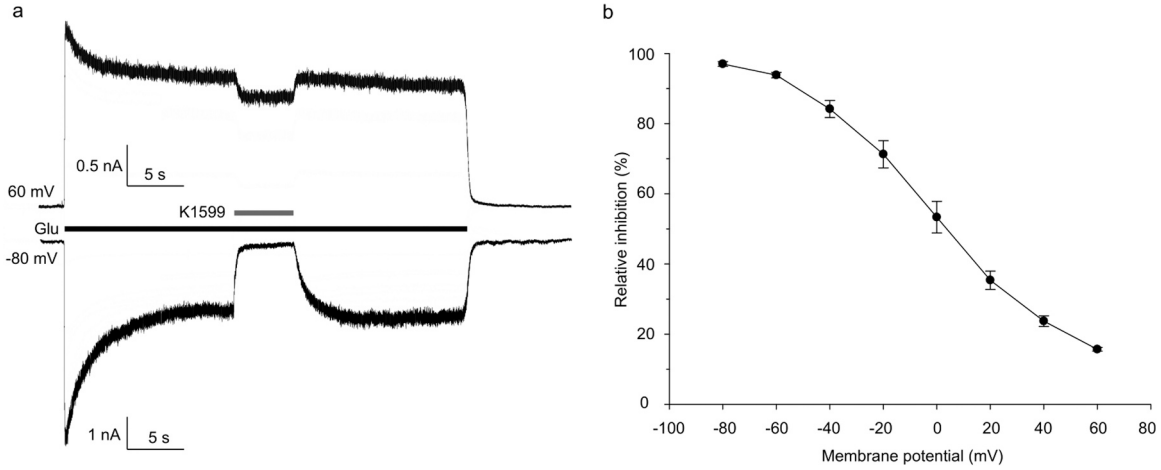

Fig. 6. Electrophysiology: Inhibition of GluN1/GluN2A receptors by K1599. Representative whole-cell patch-clamp recordings measured from HEK293 cells expressing the GluN1/GluN2A receptors held at a membrane voltage of −80 mV and +60 mV; 30 μM K1599 was applied as indicated (**a**). Graph summarizing the relative inhibition induced by 30 μM K1599, measured at the indicated membrane potentials. $n \geq 5$ cells per each condition (**b**).

reversal learning observed with K1578 and K1599 may stem from demonstrated variations in AChE inhibition levels across various brain structures. It would be interesting to investigate whether these variations were caused by differences in the distribution of the compounds to the brain structures or by other factors. Notably, a similar brain structure-dependent degree of AChE inhibition was observed with tacrine. Systemic administration of tacrine induced a relatively higher level of AChE inhibition in the striatum than in the hippocampus, while the opposite was true for another AChE inhibitor, rivastigmine [38]. Correspondingly, a lower dose of tacrine is sufficient to increase acetylcholine levels in the striatum than in the hippocampus or cortex [78]. Thus, the brain structure-dependent effects of AChE inhibitors may lead to their differing effects on individual cognitive tasks.

Whole brain AChE inhibition was not significant for any compound, underscoring the importance of assessing individual brain structures. Unexpectedly, despite lower *in vitro* IC_{50} values for (human recombinant) AChE compared to K1578 and K1599 [33], K1592 and K1594 did not inhibit AChE in any brain structure. This discrepancy may perhaps be related to differences in distribution to the brain [33], emphasizing the importance of *in situ* AChE activity assessment.

In summary, K1578 and K1599 exhibit brain structure-specific AChE inhibition, which may explain their positive effects on reversal learning and the lack of effect on spatial memory acquisition in the scopolamine-induced model. Our findings also highlight the importance of *in situ* AChE activity assessment in preclinical research in parallel with behavior experiments.

4.2. Glutamatergic effects

The second part of the study focused on the interactions of the compounds with the glutamatergic system, specifically NMDA receptors. Consistent with the literature, MK-801, a potent NMDA receptor antagonist, impaired MWM acquisition [39,79], underlining that intact NMDA receptors are crucial for learning and memory [45,46]. However, the selected dose of MK-801 did not impair reversal learning. Literature dealing with the effects of MK-801 on spatial reversal learning provides conflicting results [80–82]. Of the compounds studied, K1599 (1 mg/kg) mitigated the MK-801-induced deficit of spatial learning, possibly by competing with MK-801 on NMDA receptors.

NMDA receptor antagonists represent a heterogeneous group of compounds with diverse biological effects. NMDA receptors are essential for physiological functions including memory, but their overactivation is implicated in neuropathological processes [10]. Consequently, NMDA receptor antagonists may possess therapeutic benefits but also side effects, likely depending on their precise mechanisms of interaction with the receptor [83–86].

High-affinity open-channel blockers like MK-801 interfere with the physiological function of NMDA receptors and hence impair memory and induce other side effects like hyperlocomotion [39,87,88]. On the other hand, some open-channel blockers with moderate affinity, like memantine and 7-MEOTA, or some NMDA receptor subunit-selective antagonists may possess lower risk of side effects [32,34,83–85,89,90] and even positive impact on cognition [10,88,91]. Notably, our tacrine derivatives previously showed no side effects typical of NMDA receptor antagonists [33]. When co-administered, NMDA receptor antagonists can interact complexly, either potentiating or mitigating behavioral effects of each other [84,89]. Counterintuitively, some NMDA receptor antagonists can reverse MK-801-induced cognitive deficits [85,92].

In our study, K1599, uniquely effective against the MK-801-induced cognitive deficit, may perhaps owe its efficacy to superior brain availability compared to the other studied compounds [33]. Moreover, its NMDA receptor subunit-dependent action, namely preferential inhibition of GluN1/GluN2A over GluN1/GluN2B receptors, [33], could potentially also play a role.

Most NMDA receptors are heterotetramers containing GluN1 and GluN2A–D subunits. GluN2A and GluN2B represent the most abundant GluN2 subunits in the cognition-related structures in the adult brain [93,94]. However, they show different properties and roles [94], and their selective antagonists may exert different behavioral effects [88]. Regarding our study, MK-801 potently inhibits both GluN1/GluN2A and GluN1/GluN2B receptors [95], while our tacrine derivatives are less potent and show slight preference for either GluN1/GluN2A (K1592, K1599) or GluN1/GluN2B (K1578, K1594) receptors [33]. GluN2B-selective antagonists seem potentially appropriate as AD drugs, as these receptors are supposed to play a role in its pathophysiology [94] and contribute to neuronal injury [35,36]. In view of that, the pro-cognitive effect of the GluN1/GluN2A-preferring antagonist K1599 may seem surprising. On the other hand, long-term potentiation and hence memory processes predominantly depend on GluN2A- (rather than GluN2B-) containing receptors [96,97]. Therefore, we hypothesize that preferential competition of K1599 with MK-801 for binding on GluN2A-containing receptors may perhaps contribute to mitigation of cognitive deficit. Nevertheless, it is not clear whether the GluN2A-preference of K1599 is indeed responsible for its pro-cognitive effect.

Next, we studied the effects of the compounds on the locomotion of both intact animals and animals with MK-801-induced hyperlocomotion [83,89] in the open field. Compounds that decreased locomotion in intact animals (K1578 at 5 mg/kg, K1592 at 1 mg/kg, K1594 at 5 mg/kg) also mitigated MK-801-induced hyperlocomotion, suggesting a non-specific effect not necessarily mediated via NMDA receptors. AChE inhibitors can induce similar effects, as known from the literature [50,98,99] and observed in our laboratory with the AChE inhibitor donepezil (unpublished data). Therefore, the AChE-inhibitory properties of the compounds may be perhaps responsible.

K1578 (1 mg/kg) and K1599 (5 mg/kg) displayed interesting interactions with MK-801, potentially reflecting their subunit-dependent NMDA receptor inhibition. The GluN2B-preferring compound K1578 slightly potentiated MK-801-induced hyperlocomotion at a dose that did not affect locomotion in intact animals (1 mg/kg). Interestingly, a similar effect was described with another GluN2B-preferring NMDA receptor antagonist, ifenprodil [89]. On the other hand, K1599 mitigated the MK-801-induced hyperlocomotion at a dose that did not affect locomotion in intact animals (5 mg/kg). A similar effect was described with 7-MEOTA and interpreted as competition of 7-MEOTA and MK-801 for the binding site on NMDA receptors [32]. The effect of K1599 and 7-MEOTA may be perhaps related to their GluN2A-preference [32,33], as the locomotion-stimulating effect of MK-801 seems to be mediated largely via inhibition of GluN2A-containing NMDA receptors [95]. The open field and MWM results seem to be consistent with the notion that K1599 affects NMDA receptors in a way that does not impede their physiological function, but by competing with MK-801 for binding at NMDA receptors, K1599 mitigates the detrimental effects of MK-801.

The findings from our *in vitro* electrophysiology study align with this concept. Whole-cell patch-clamp measurements on transfected HEK293 cells expressing rat GluN1/GluN2A receptors indicated that K1599 inhibits the GluN1/GluN2A receptors at all studied membrane potentials, with a more pronounced effect at the negative membrane potentials. This conclusion is in agreement with our previous data [33]. While it is technically challenging to directly show competition between K1599 and MK-801 using *in vitro* electrophysiology because of the "irreversible" nature of the MK-801 block [100], we conclude that both K1599 and MK-801 likely act as open-channel blockers. Therefore, they could compete for the same binding site within the ion channel region of the GluN1/GluN2A receptor *in vivo*.

4.3. The dual effect of K1599: From structural modifications back to 7-MEOTA-like compounds

To sum up, K1599 demonstrated the most favorable effects among the compounds studied, mitigating cognitive deficits induced by disruptions to both the cholinergic and glutamatergic systems at a fixed

dose (1 mg/kg). This aligns with its intended dual cholinesterase- and NMDA receptor-inhibitory effect.

Interestingly, of the compounds studied, the favorable compound K1599 possesses the most similar structure and affinities for target proteins as 7-MEOTA. K1599 differs from 7-MEOTA only by the smaller size of the cycloalkyl moiety attached to the aromatic region. Both 7-MEOTA [28,32] and K1599, in contrast with the other compounds in the current study, showed beneficial *in vivo* effects in the glutamatergic model, consistent with NMDA receptor inhibition. K1599 and 7-MEOTA display balanced IC_{50} values for AChE, BChE, GluN1/GluN2A, and GluN1/GluN2B receptors (at – 60 mV), differing by no more than one order of magnitude [32,33], which is considered one of the fundamental features of multi-target drugs [12,14]. The *in vitro* IC_{50} values of K1599 exhibit following relationship: GluN1/GluN2A < AChE < BChE < GluN1/GluN2B (4.16, 8.22, 10.6, and 14.56 μM, respectively, with inhibition of NMDA receptors measured at the membrane potential of – 60 mV) [33]. This specific order and narrow range of IC_{50} values for target proteins seem optimal for favorable *in vivo* effects. Interestingly, both K1599 and 7-MEOTA show slight preference for GluN1/GluN2A over GluN1/GluN2B receptors [32,33]. Besides, the higher brain availability of K1599 compared to the other compounds from the current study [33] may contribute to its superior effects when given in equal doses.

On the other hand, the compounds K1578, K1592, and K1594 were not beneficial. They differ from K1599 by different substituents at the core moiety and the size of the cycloalkyl moiety. Contrary to K1599, these compounds display preferential inhibition of cholinesterases over NMDA receptors [33]. K1578, with a chlorine atom substitution in the position identical to the methoxy substitution in 7-MEOTA, was effective only in the cholinergic model. K1592 and K1594, displaying increased inhibitory potency for AChE or both AChE and BChE *in vitro* [33], were not considerably effective in either model. Regarding K1599, it is noteworthy that its dual effect was manifested only at one of the two doses tested, highlighting the importance of including multiple doses of compounds in preclinical studies. To sum up, our results indicate that tacrine derivatives with structural and functional similarity to 7-MEOTA may be especially promising in the development of AD drugs with dual *in vivo* effects.

4.4. Study limitations

The study has several limitations. Firstly, the use of the MK-801-induced model lacks a direct link to AD; instead, it served as a pharmacological tool to explore NMDA receptor-mediated behavioral effects. Consequently, translating our findings, such as the role of NMDA receptor subtype preference of the compounds, to AD patients remains uncertain. Future testing of the neuroprotective effect of K1599 in another, more disease-relevant model of glutamatergic pathology, namely the NMDA-induced model of excitotoxic hippocampal neurodegeneration [101], may provide additional information. Moreover, validating the therapeutic potential of K1599 in a transgenic amyloid animal model of AD would be helpful.

Secondly, although the scopolamine- and MK-801-induced models were employed to investigate AChE- and NMDA receptor-mediated effects, respectively, it is essential to recognize the close interaction between both neurotransmitter systems (see [10]). It was reported that in some cases, cholinergic agents can indirectly affect the glutamatergic system [50,99,102], while glutamatergic agents can influence the cholinergic system [19,22,103,104]. Tacrine derivatives' effects in both models might hence result from intricate favorable cholinergic and glutamatergic interactions. Moreover, although the behavioral effects align with the known mechanisms of action of the tacrine derivatives (i.e., glutamatergic and cholinergic), the involvement of other neurotransmitter systems or off-target effects cannot be ruled out, as these were not controlled for in our study.

Lastly, our compounds vary not only in affinities for target proteins but also in brain availability, potentially influencing behavioral outcomes and complicating their comparability. While intracerebroventricular administration could address this issue, it is deemed inappropriate for the extensive screening study due to invasiveness, clinical irrelevance, and time demands.

5. Conclusions

Our comprehensive *in vivo* study delved into the effects of tacrine derivatives, functioning both as cholinesterase inhibitors and NMDA receptor antagonists, utilizing animal models reflective of cognitive deficits arising from cholinergic or glutamatergic dysfunction. K1599 emerged as the most promising compound, demonstrating pro-cognitive efficacy at a consistent dose across both models, hence confirming its dual *in vivo* effect. We conclude that tacrine has the potential for the development of derivatives with dual *in vivo* pro-cognitive effects. Compounds similar in structure and function (IC_{50} values to target proteins) to K1599 appear to be particularly promising. Deciphering the structural and functional attributes of tacrine derivatives associated with optimal *in vivo* pro-cognitive effects holds potential for advancing the development of dual compounds as promising therapeutics for AD.

Competing interests

The authors declare that they have no competing interests.

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CRediT authorship contribution statement

Jan Korabecny: Resources, Funding acquisition. **Martin Horak:** Funding acquisition. **Karel Vales:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Ondrej Soukup:** Funding acquisition. **David Kolar:** Writing – review & editing, Investigation, Formal analysis. **Marketa Chvojkova:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Conceptualization. **Lada Cejkova:** Investigation. **Katarina Kovacova:** Investigation. **Kristina Hakenova:** Investigation. **Anna Misiachna:** Investigation. **Lukas Gorecki:** Resources.

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.

Data Availability

Data will be made available on request.

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