Antagonizing α7 nicotinic receptors with methyllycaconitine (MLA) potentiates receptor activity and memory acquisition

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ABSTRACT

α7 nicotinic acetylcholine receptors (α7nAChRs) have been targeted to improve cognition in different neurological and psychiatric disorders. Nevertheless, no α7nAChR activating ligand has been clinically approved. Here, we investigated the effects of antagonizing α7nAChRs using the selective antagonist methyllycaconitine (MLA) on receptor activity in vitro and cognitive functioning in vivo. Picomolar concentrations of MLA significantly potentiated receptor responses in electrophysiological experiments mimicking the in vivo situation. Furthermore, microdialysis studies showed that MLA administration substantially increased hippocampal glutamate efflux which is related to memory processes. Accordingly, pre-tetanus administration of low MLA concentrations produced longer lasting potentiation (long-term potentiation, LTP) in studies examining hippocampal plasticity. Moreover, low doses of MLA improved acquisition, but not consolidation memory processes in rats. While the focus to enhance cognition by modulating α7nAChRs lies on agonists and positive modulators, antagonists at low doses should provide a novel approach to improve cognition in neurological and psychiatric disorders.

1. Introduction

α7 nicotinic acetylcholine receptors (α7nAChRs) are cation permeable ligand-gated ion channels that are expressed abundantly in the mammalian brain with a high permeability to Ca²⁺. α7nAChRs are homomeric receptors constituted by five identical subunits arranged in a barrel-like manner and keeping in its center the ionic pore. These receptors have been implicated in the modulation of cognitive functions like attention and episodic memory (see for example [1]). α7nAChRs are located pre-, post- and non-synaptically [2] and modulate the release of glutamate [3,4], GABA [5] and dopamine [6] by influencing intracellular Ca^{2+} concentrations. Furthermore, α7nAChRs are directly involved in hippocampal long-term potentiation (LTP) [7], a putative

cellular mechanism underlying learning and memory [8]. LTP can be divided into two phases; the transient early phase of LTP (E-LTP) and the long-lasting form which is called late phase LTP (L-LTP). The latter is dependent on protein synthesis and induces longer lasting plastic changes via for instance phosphorylation of the cAMP-Responsive Element Binding (CREB) transcription factor [9].

The disease linkage of the α7nAChR has been reviewed recently [10,11] and shows associations to multiple disorders with cognitive deficits, including schizophrenia and Alzheimer's disease (AD). Activation of α7nAChR was shown to improve cognitive function in both animal (see for example [12,13]) and human studies (see for example [14–16]). The main cognitive improvements relate to memory, which correlates with the high level of expression of α7nAChRs in the

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prefrontal cortex and hippocampus. Hence, different α7nAChR specific (partial) agonists have been investigated for their potential to improve memory and attention disorders encountered in, for instance, schizophrenia and AD (see for example [1]). However, no α7nAChR selective compound has as of yet been approved for this indication. In 2015, all phase 3 clinical trials of EVP-6124 (encenicline) were terminated due to unwanted side-effects (AD) or insufficient cognitive improvements (schizophrenia). Also, a recent meta-analysis indicated that agonizing α7nAChRs is not a robust treatment for cognitive dysfunction in AD and schizophrenia [17].

Low dose monotreatment with the selective α7nAChR antagonist methyllycaconitine (MLA) has sporadically been reported to improve cognition in animals [18,19]. Furthermore, MLA has been shown to facilitate LTP induction in hippocampal region CA1 in rats [20]. MLA is a norditerpenoid alkaloid produced by many species of Delphinium (larkspurs) [21] and is selective for the α7nAChR over other nAChRs or ligand-gated ion channels [21–25]. MLA is often used because of its ability to potently antagonize the α7nAChR in animal models to show that putative α7nAChR agonists indeed work through the α7nAChR mechanism (see for example [26]). To our knowledge no studies specifically investigating the mechanism behind the effects of MLA on α7nAChR functioning and cognitive functioning, have been published.

The objective of the current study was to investigate the effect of low dose administration of the selective α7nAChR antagonist methyllycaconitine (MLA) on receptor activity in vitro, glutamate release in vivo, LTP ex vivo and memory function in vivo. It was investigated whether MLA can enhance receptor activity by performing electrophysiological experiments that mimic in vivo neuronal signaling. Since hippocampal glutamate efflux is modulated by Ca^{2+} levels and has been associated with object memory processes in rats [27], microdialysis studies were performed measuring glutamate efflux in rat hippocampi after administering different doses of MLA. Furthermore, LTP electrophysiological studies were performed to investigate concentration-dependent effects of MLA on hippocampal LTP. Lastly, memory acquisition and consolidation after MLA treatment was assessed in male rats using the object recognition task (ORT).

2. Materials and methods

All procedures were designed to minimize potential discomfort of the animals during the behavioral experiments. All experimental procedures were approved by the local ethical committees for animal experiments according to local governmental guidelines.

2.1. Reagents

Methyllycaconitine citrate salt hydrate (MLA) was obtained from Research Biochemicals International/Sigma-Aldrich and Tocris (#13A/84415). Acetylcholine-chloride was obtained from Sigma-Aldrich (A6625, Lot# BCB3758V).

2.2. Animals

For the electrophysiological receptor functioning studies, oocytes were harvested from adult *Xenopus laevis* females. Frogs were housed socially in groups of maximum eight animals. Animals were kept in water tanks of 50 L while maintaining the water temperature at 19–22 °C. Water tanks were placed in a temperature-controlled room of 20–23 °C with protection from direct sunlight. Once per week, frogs were fed and water quality was assessed by measuring pH, nitrate, nitrite, chlorine, alkalinity and water hardness.

For the microdialysis studies, a total of twenty-one 3-month-old male Sprague Dawley (SD) rats (Charles River, Calco, Italy) were used. Animals were maintained on a 12/12-h light/dark cycle in temperature (21 ± 1 °C)- and humidity (50%)-controlled rooms; food and water were available ad libitum.

For electrophysiological LTP studies, we used a total of twenty-four C57BL/6J male mice, 3–5-month-old, obtained from a breeding colony housed in the animal facility of the University of Catania. Housing conditions of the mice were the same as for rats, except that they were housed socially with five animals per cage.

For the ORT studies, a total of thirty-six 3–4-month-old male Wistar rats (Charles River, Sulzfeld, Germany) were used. The animals were housed individually in temperature (21 ± 1 °C)- and humidity (60 ± 10%)-controlled rooms; food and water were available ad libitum. The animals were maintained under a reversed 12/12-h light/dark cycle. During the dark period, fluorescent red tubes provided a constant illumination (2 lx). Behavioral experiments were performed in the dark period in the same room where the rats were housed. During testing the test room was dimly lit by a small lamp (25 W), located in the corner of the room.

2.3. Electrophysiology: receptor activity

Oocytes were harvested from mature *Xenopus laevis* females and were prepared, injected with cDNA, and recorded using standard procedures [26,28]. On the day after dissociation, oocytes were injected with 0.2 ng of cDNA expression vector, coding for the human α7nAChR subunit, in their nucleus using an automatic injection system (RoboInject, Multi Channel Systems) [28]. Subsequently, oocytes were maintained in Barth's medium containing 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 10 mM HEPES, 0.82 mM MgSO₄·7H₂O, 0.33 mM Ca(NO₃)₂·4H₂O, 0.41 mM CaCl₂·6H₂O, and complemented with penicillin (100 U/ml) and streptomycin (100 U/ml) at 18 °C for 2–7 days to allow for proper gene transcription.

Recordings were made using a two-electrode voltage-clamp configuration (HiClamp, Multi Channel Systems). Electrodes made from borosilicate (0.5 and 2.5 MΩ) were filled with 3 M KCl. Throughout experiments membrane potentials were maintained at −80 mV and cells were superfused with OR2 (oocyte ringer) containing 88 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, adjusted to pH 7.4 with NaOH. Concentration-dependent effects of MLA incubation (1, 3, 10 and 100 pM) were by tested by measuring currents evoked by irregularly co-applied ACh pulses (5 s, 40 μM) every 3 min [13,26]. Stock solutions of acetylcholine-chloride (Sigma-Aldrich, A6625, Lot# BCB3758V) and MLA (Tocris, #13A/84415) (dissolved in bi-distilled water) were diluted in OR2 immediately before use. Data acquisition and analysis was performed using Matlab (Mathworks Inc.) and Excel (Microsoft) software.

2.4. Microdialysis

Rats were anaesthetized with chloral hydrate (400 mg/kg), placed in a stereotaxic frame (David Kopf Instruments) and implanted with microdialysis probes transversely positioned into the dorsal hippocampi at the following coordinates: AP = +3.8, H = +6.5 from the interaural line [29]. The surgical procedure was performed as reported elsewhere [30,31]. After surgery, rats were allowed to recover in their home cages for 24 h. The day after surgery, rats were placed into observation cages, and the probes were infused at a flow rate of 5 μL·min⁻¹ (CMA/100 microinjection pump, CMA Microdialysis) with modified Ringer's medium containing (in mM): NaCl 145, KCl 3, MgCl2 1, CaCl2 1.26, buffered at pH 7.4 with 2 mM phosphate buffer. Following a 1 h stabilization period, consecutive samples were collected every 10 min. MLA was i.p. injected (0.01, 0.1, 1.0 and 10 mg/kg) after five control samples had been collected to estimate basal glutamate levels. At the end of the experiment, rats were euthanized by anesthetic overdose and the correct position of the probe was verified by optical examination of the fiber tract. Animals presenting hemorrhagic lesions or with probe track outside the target region were excluded from the results. Microdialysis samples were stored at −30 °C until HPLC analysis for amino acid determination as previously described [32].

2.5. Electrophysiology: hippocampal LTP

Electrophysiological recordings were performed as previously described [33]. Briefly, transverse hippocampal slices (400 μm) were cut and transferred to a recording chamber where they were maintained at 29 °C and perfused with ACSF (flow rate 2 ml/min) continuously bubbled with 95% O_2 and 5% CO_2. The ACSF composition was composed of the following (in mM): 124.0 NaCl, 4.4 KCl, 1.0 Na_2HPO_4, 25.0 $NaHCO_3$, 2.0 $CaCl_2$, 2.0 $MgCl_2$, and 10.0 glucose. Field extracellular recordings were performed by stimulating the Schaeffer collateral fibers through a bipolar tungsten electrode and recording in CA1 stratum radiatum with a glass electrode filled with ACSF. A 15 min baseline was recorded every minute at an intensity that evoked a response approximately 35% of the maximum evoked response. Early-LTP was induced by a weak tetanus (4 pulses at 100 Hz, with the bursts repeated at 5 Hz and one tetanus of 10-burst trains) [33–37]. MLA (30, 100 and 300 nM) was given for 10 min either right before or 10 min after tetanus to reflect behavioral treatment conditions [33]. Responses were recorded for 2 h after tetanization and measured as field excitatory postsynaptic potentials (f-EPSP) slope expressed as percentage of baseline.

2.6. Object recognition task (ORT)

The ORT training and testing procedures were performed as described elsewhere [38,39]. The apparatus and objects are identical to those described previously [39]. All compound solutions were prepared on the day of experimental testing and administered i.p. by a blinded experimenter in a counterbalanced design. MLA was prepared in saline at an injection volume of 1 ml/kg. Doses were based on the weight of the salt of the compound. Because rats were retested with different compound doses, test sessions were scheduled to allow at least a two-day wash-out period.

2.7. Memory acquisition experiments

The effect of MLA on the acquisition processes of long-term memory was investigated using a 24 h interval between the ORT learning- and test-trials (T1 and T2, respectively). The memory acquisition processes were targeted by administering the compounds before T1 in the ORT [33,39]. Untreated Wistar rats normally show no significant object memory performance after 24 h (see for example [26]). MLA (0.001–1.0 mg/kg, i.p.) was administered 30 min before T1. The administration time of MLA was chosen to correspond with the time for the compound to reach peak brain concentrations [40]. MLA was also tested in a 1 h retention interval. Untreated Wistar rats normally show significant object memory after a 1 h retention interval (see for example [26]). So, in this experiment the ability of MLA to reverse significant memory using a 1 h retention interval was tested. Doses of 0.1–1.0 mg/kg MLA (i.p.) were administered 30 min before T1. For the control treatment, animals received the vehicle of MLA (saline). In both paradigms, all 18 animals received each treatment (within-subjects design).

2.8. Memory consolidation experiments

The effect of MLA on the consolidation processes of long-term memory was also investigated using a 24 h interval between the ORT trials. The memory consolidation processes were targeted by administering the compounds after T1 in the ORT [33,39]. Optimum doses of MLA (0.03–0.1 mg/kg, i.p.) were administered 4–10 min after T1 to specifically target memory consolidation processes [33]. The optimum doses of MLA were based on the memory acquisition experiments. Like in the previous experiments, for the control treatment animals received saline. All 18 animals received each treatment (within-subjects design).

2.9. Statistical analyses

For the electrophysiological receptor activity studies, normalized evoked currents from experimental conditions were compared to the control condition by means of performing two-tailed Student's *t*-tests assuming unequal variances.

In the microdialysis studies, all data were expressed as percentages of the mean basal value for each animal. The mean basal value has been calculated as the average of the first five microdialysis fractions before the injection (fraction 6). Data were analyzed using one-way ANOVA. Significant main effects were followed by two-tailed paired-samples *t*-tests between fraction 5 (the final baseline) and all subsequent collections. In case of a significant main effect, two-tailed independent samples *t*-tests were also performed between the effective MLA dose and the vehicle/control group per fraction. In all analyses, an α level of 0.05 was considered significant.

Data from hippocampal LTP electrophysiology studies were statistically analyzed by performing one-way ANOVA with repeated measures comparing 120 min of recording after tetanus (Systat software).

The ORT provides measures for exploration time and discrimination [39]. The basic measures are the times spent by the animals exploring an object during both the learning and the test trial (T1 and T2, respectively). The times spent exploring the two identical objects in T1 and two different objects in T2 are represented by 'e1' and 'e2', respectively. The d2 index is a relative measure of discrimination corrected for exploratory activity (e2) [39]. The d2 index can range from −1 to 1, with −1 or 1 indicating complete preference for the familiar or novel object, respectively and 0 signifying no preference for either object. Object recognition performance was assessed by performing two-tailed one-sample *t*-statistics. This way it could be assessed per treatment condition whether the d2 index differed significantly from zero (chance level). Effects on e1, e2 and d2 between the different treatment conditions were assessed by repeated measures ANOVA. When the overall ANOVA was significant, post hoc Bonferroni *t*-tests (all pairwise comparisons) were used. Animals with insufficient exploration times (< 7 s) were excluded from the dataset [39].

3. Results

3.1. Electrophysiology: receptor activity

Neuronal signaling in vivo is rather irregular with bursts of activity separated by silent periods. To mimic this signaling in our heterologous system, the effect of irregular co-application of the natural agonist ACh on receptor activity was investigated during continuous incubation with MLA [13,26]. During a stabilization period in absence of MLA, currents were evoked by brief ACh pulses (5 s, 40 μM) every 3 min. Then, ACh was irregularly co-applied during continuous incubation with MLA. At the end of the protocol, currents were regularly evoked during a recovery phase without MLA (Fig. 1a). Picomolar concentrations of MLA (1 to 100 pM) were tested using this protocol. The maximal amplitudes of evoked currents are shown as mean ± SEM (Fig. 1b). Data were normalized to the average current evoked during the stabilization period. The same protocol was conducted in absence of MLA to control for effects on current amplitudes by the protocol itself. Per pulse, MLA conditions were compared to control by performing two-tailed Student's *t*-tests assuming unequal variances (α = 0.05). For the 1 and 3 pM conditions a trend was found for the irregular ACh pulse after the first 'silent period' (P = 0.058 and P = 0.076, respectively). A significant augmentation of current amplitude evoked at the end of MLA incubation was found for 1 and 3 pM MLA (P = 0.045 and P = 0.015, respectively), which persisted after MLA was removed for the 3 pM MLA condition (P = 0.047). As expected for a competitive antagonist, at the highest concentration MLA (100 pM) inhibited the ACh-evoked current as evident for the second and third pulse applied at the beginning of MLA application (P = 0.007 and P = 0.005,
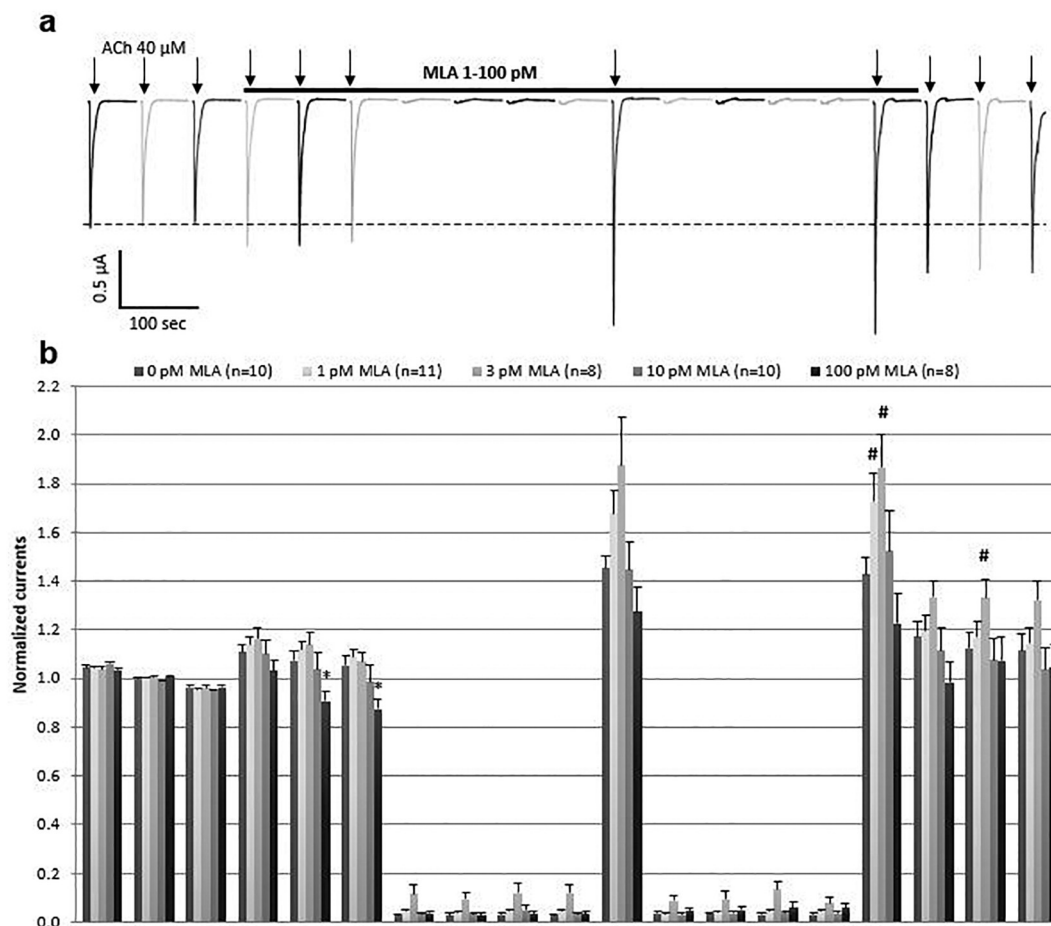
Fig. 1. Electrophysiological experiments investigating the effect of MLA on α7nAChR activity. (a) Protocol used to investigate the effect of continuous incubation with MLA concentrations (horizontal bar) and irregular co-application of the natural agonist ACh (arrows) on receptor activity. (b) Picomolar concentrations of MLA (1–100 pM) were tested on human α7nAChRs. Current amplitudes are presented as mean ± SEM and are normalized to the mean of the control measurements before MLA application. Low concentrations MLA (1–3 pM) significantly potentiated receptor responses at the end of MLA incubation, while the significant inhibiting effect by 100 pM MLA at the start of MLA incubation ceased over time. Since potentiation was observed even in absence of MLA, results were statistically compared to the control condition per pulse by two-tailed Student's *t*-tests (unequal variations assumed; *p < 0.01, #p < 0.05, N = 8–11 oocytes).

respectively). Interestingly, this inhibitory effect ceased over time. Overall, responses became less inhibited or even potentiated as MLA concentrations incubated longer, suggesting a time-dependent effect.

3.2. Microdialysis

Glutamate efflux was increased after 1.0 mg/kg MLA administration ($F_{16,49} = 4.32$; P < 0.001). These effects were seen from dialysate 9 onwards, extending for at least up to 2 h (fraction 17). Fraction 9 corresponds to 30 min after MLA administration, which resembles the injection/testing regimen of the ORT study (Fig. 2). ANOVA also showed an effect in the vehicle condition ($F_{16,45} = 2.15$; P = 0.023). Post hoc analysis showed that the last dialysate was significantly lower (P = 0.042) when compared to the dialysate at fraction 5 (baseline). Because of this effect, two-tailed independent samples *t*-tests were also performed to check whether 1.0 mg/kg MLA also differed from the vehicle/control condition per fraction. The glutamate efflux after 1.0 mg/kg MLA was significantly higher than the vehicle/control condition from fraction 10 (40 min after the injection) onwards.

3.3. Electrophysiology: hippocampal LTP

Electrophysiological experiments were performed by using a weak tetanus to produce *E*-LTP, which is a transient potentiation, at Schaffer collateral-CA1 synapses in hippocampal slices. A 10 min perfusion with

a low concentration of MLA (100 nM) before tetanus produced a longer lasting potentiation (219.87 ± 32.45% vs. 131.68 ± 4.31% of baseline slope, N = 7 slices; $F_{1,12} = 14.306$, P = 0.003; Fig. 3a), whereas no changes in E-LTP were recorded when MLA was administered after tetanus (123.20 ± 11.18% of baseline slope, N = 6 slices; $F_{1,11} = 1.72$, P = 0.216; Fig. 3a). A dose-response curve for the effect of pre-tetanus administration of MLA on LTP showed that both 30 nM and 100 nM MLA concentrations were able to enhance potentiation (30 nM: 197.56 ± 25.98% of baseline slope, N = 6 slices; $F_{1,11} = 5.18$, P = 0.044; Fig. 3b), whereas a higher concentration (300 nM) did not modify LTP (113.10 ± 5.41% of baseline slope, N = 6 slices; $F_{1,11} = 0.55$, P = 0.47; Fig. 3b).

3.4. ORT: memory acquisition processes

In the 1 h retention interval, no differences were found between treatment conditions on the level of exploration in T1 (e1, $F_{3,51} = 0.27$; P = 0.844) or T2 (e2, $F_{3,51} = 0.90$; P = 0.447). The d2 indices differed between treatment conditions ($F_{3,51} = 7.33$; P < 0.001). Post hoc analyses revealed significantly lower object discrimination in the 1.0 mg/kg MLA condition (P = 0.003) when compared to the vehicle condition. Two-tailed one-sample *t*-statistics showed that the discrimination performance was significantly different from zero in the vehicle, 0.1 and 0.3 mg/kg MLA conditions (Fig. 4a). In the 24 h retention interval, exploratory behavior did not differ between treatment
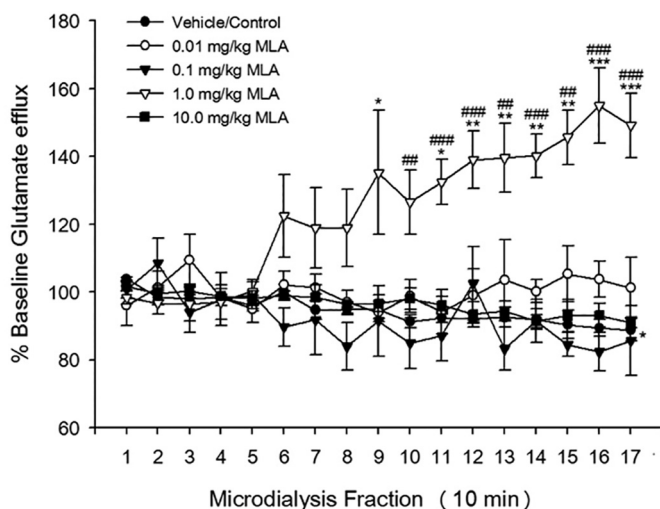
Fig. 2. Glutamate efflux at baseline and after treatment with 0–10 mg/kg MLA (i.p.) at dialysate fraction 6. Efflux was significantly increased from 30 min onwards after 1.0 mg/kg MLA treatment (dialysate fraction 9) when compared to both baseline level (dialysate fraction 5) and to the vehicle/control condition per fraction point. Data represent mean ± SEM. Differences from dialysate fraction 5 (baseline) (ANOVA): *p < 0.05; **p < 0.01; ***p < 0.001; Differences from vehicle/control group (two-tailed independent samples *t*-tests): #p < 0.05; ##p < 0.01; ###p < 0.001, N = 3–5 rats per treatment condition.

(e1, $F_{7,119}$ = 1.84; P = 0.087 and e2, $F_{7,119}$ = 1.59; P = 0.146). Discrimination performance (d2) differed between treatment conditions ($F_{7,119}$ = 4.37; P = 0.000). Post hoc analyses revealed significantly higher object discrimination in the 0.003 mg/kg (P = 0.041), 0.03 mg/kg (P = 0.020) and 0.1 mg/kg MLA (P = 0.002) conditions when compared to the vehicle condition. Two-tailed one-sample *t*-statistics showed that the discrimination performance was significantly different from chance level in the dosage range from 0.003 to 0.3 mg/kg MLA (Fig. 4b).

3.5. ORT: memory consolidation processes

In the 24 h retention interval, no differences were found between treatment conditions on the level of exploration in T1 (e1, $F_{2,34}$ = 2.71; P = 0.081). In T2, a difference was found for the exploration times between the different treatment conditions (e2, $F_{2,34}$ = 7.34; P = 0.002). Post hoc analyses revealed significantly lower exploration times in T2 for the 0.1 mg/kg MLA condition when compared to both the vehicle (P = 0.009) and the 0.03 mg/kg MLA condition (P = 0.029). Furthermore, the d2 indices did not differ between treatment conditions ($F_{2,34}$ = 0.13; P = 0.876). Likewise, two-tailed one-sample *t*-statistics showed that none of the discrimination performances were significantly different from chance level in any of the experimental conditions (Fig. 4c).

4. Discussion

In the current study, we showed that antagonizing α7nAChRs using the selective antagonist MLA counterintuitively potentiated receptor activity. Picomolar concentrations of MLA (1–3 pM) led to a significantly enhanced receptor response upon agonist co-application. To describe the plausible functioning of ligand-gated ion channels, *the allosteric theory* of ion channel functioning was developed [41,42] and predicted that ion channel receptors can exist in different conformational states (for a review see: [43]). Furthermore, these receptors can undergo spontaneous transitions between these conformational states [44]. To get an understanding of nAChR functioning, at least three
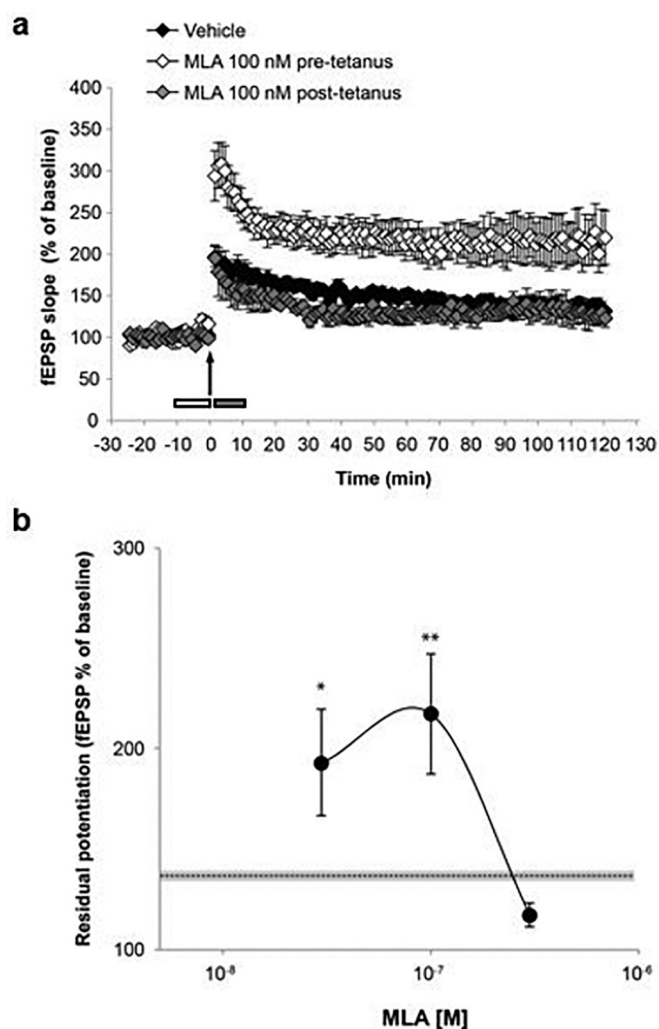
Fig. 3. Effects of the α7nAChR antagonist MLA on hippocampal LTP. (a) Ten minutes perfusion of hippocampal slices with a low dose (100 nM) MLA before, but not after tetanus increased levels of potentiation when compared to vehicle treated slices. The arrow indicates tetanus delivery (one 10-burst stimulation – weak tetanus) and the horizontal bars indicate the period during which MLA was added to the bath solution (white, pre-tetanus; grey, post-tetanus). (b) Dose-response curve for the effect of pre-tetanus administration of MLA on CA1-LTP indicating that the α7nAChR antagonist had an enhancing effect at 30 and 100 nM, whereas it did not affect potentiation at 300 nM. The dotted line and the shaded area around it correspond to the amount of potentiation in vehicle-treated slices. Residual potentiation was calculated by averaging the last 5 min of LTP. Data are represented as mean ± SEM. One-way ANOVA: *p < 0.05; **p < 0.01, N = 6–7 hippocampal slices.

conformational receptor states must be taken into account. These states are: Resting, Active and Desensitized. Each conformational state displays a different affinity for different ligands [43,45]. Low doses of α7nAChR antagonists could theoretically alter the rate of desensitization or re-sensitization of these receptor ion channels. The orthosteric binding domain of the nAChR is at the interface between the α-subunit and the neighboring subunit [46], leading to as many as five functional agonist binding sites for the homopentameric α7nAChR. nAChRs generally require occupancy of two agonist binding sites (by ACh or other agonist) to achieve channel opening [47], leaving in the case of α7nAChRs three additional sites on this homopentameric receptor available to bind antagonist. It is plausible that from these binding sites, the desensitization of the receptor could be prevented or delayed. In addition, it cannot be ruled out that incubation of MLA may alter the receptor's affinity to bind other ligands. Studies demonstrated that
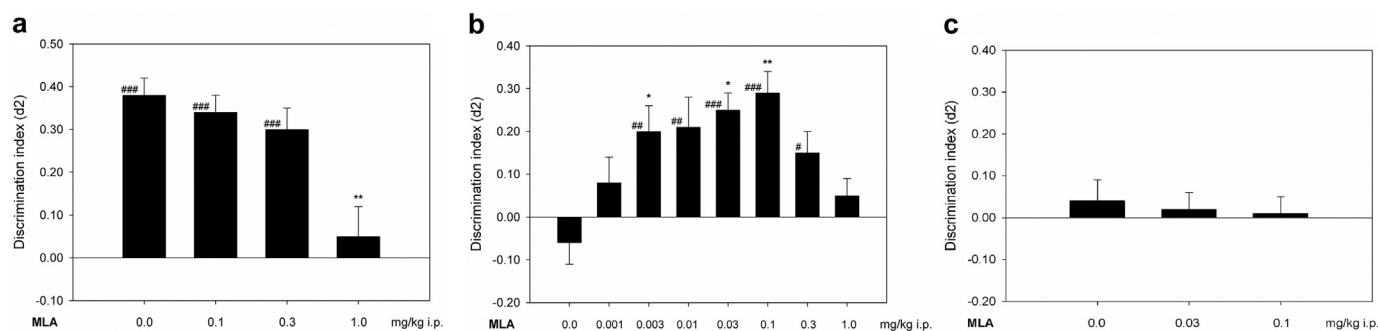
Fig. 4. ORT acquisition and consolidation studies with the α7nAChR antagonist MLA. (a) Acquisition-memory effects of MLA (i.p. administered 30 min *before* T1) in a 1 h retention interval ORT (mean ± SEM). When compared to vehicle, MLA (1.0 mg/kg) induced a short-term memory deficit in this paradigm. (b) Acquisition-memory effects of MLA (i.p.) on the d2 index using a 24 h retention interval (mean ± SEM). Compared to vehicle, MLA (0.003, 0.03 and 0.1 mg/kg administered 30 min *before* T1) prevented loss of long-term memory. (c) Consolidation-memory effects of MLA (i.p.) on the d2 index using a 24 h retention interval (mean ± SEM). When compared to vehicle, optimum doses of MLA (0.03 and 0.1 mg/kg administered 4–10 min *after* T1) did not prevent loss of long-term memory. Differences from vehicle (ANOVA): *p < 0.05; **p < 0.01. Differences from chance performance (two-tailed one-sample *t*-tests): #p < 0.05; ##p < 0.01; ###p < 0.001, N = 17–18 rats per treatment.

prolonged incubation of MLA increased the fraction of high-affinity α4β2 nAChRs [48]. Although this study used a relatively high MLA concentration, its low affinity for α4β2 nAChRs could have led to functional concentrations comparable to those that have been used in the current studies on α7nAChRs.

Another possible mechanism is based on the possible receptor occupancy configurations of MLA in combination with ACh and their effect on the receptor's functional properties. It has been demonstrated that the degree of channel activation of the pentameric α7nAChR is dependent on the binding stoichiometry of ACh [49]. It was shown that agonist binding of two consecutive binding sites induced slow activation, while binding two non-consecutive sites led to rapid activation and receptor desensitization. Binding of a third non-consecutive site is thought to maximally stabilize receptor opening, hence evoking the largest responses. Moreover, a similar relationship between the number of binding sites and receptor functionality has recently been shown for α4β2 nAChRs [50]. Rayes et al., found the profile of receptor activation for either three or five bound sites to be indistinguishable. This implicates the importance of two or three available binding sites for receptor activation. Accordingly, prototypic heteromeric ligand-gated ion channels like muscle nAChRs and the GABA_A receptor are known to possess only two non-consecutive binding sites while still remaining functional [51]. Binding of MLA may influence the ability of ACh in binding the necessary non-consecutive sites. If one or two MLA molecules are bound per receptor, there will always remain a pair of non-consecutive sites to be bound by ACh to induce receptor activation. Once MLA occupies three sites, two sites remain available to ACh, which can either be consecutive or non-consecutive. In any case, the ability to maximally stabilize channel opening by a third bound ACh molecule is lost, leading to a decrease of the response. Binding four MLA molecules will inhibit ACh in binding two (non-)consecutive sites, profoundly decreasing the probability of receptor activation. Importantly, results from an earlier study led to the assumption that binding of a single MLA molecule is sufficient to prevent channel opening [52]. This appears in contrast with the current study, yet these earlier findings might, with our current knowledge, alternatively be explained by assuming that binding of a fourth MLA molecule will prevent receptor activation rather than a single molecule.

Although the current method only indicates macroscopic currents, it can be expected that most receptors will have bound the same amount of MLA molecules at a particular concentration. Occupancy of two or three sites by MLA can each exist in two different stoichiometries. Hypothetically, over time the ratio between these stoichiometries might change towards the more favourable configurations containing non-consecutive free sites which can evoke larger currents. This could

explain the time-dependent effects observed in our receptor activity experiments (Fig. 1b). Thus, longer incubation by MLA will leave only the non-consecutive sites available to ACh resulting in the largest response. Additionally, blocking only one or two sites by MLA is expected not to influence receptor activity itself but since agonist molecules are prevented from binding all five sites, the receptor fraction that can be activated per agonist concentration is increased and/or receptor opening is stabilized which results in a larger macroscopic current. Next studies should indicate the exact mechanism by which MLA potentiates α7nAChR currents and whether these actions are observed using other antagonists as well as in combination with partial agonists.

Our microdialysis studies showed that 1.0 mg/kg MLA led to a significant increase of glutamate efflux 30 min after administration that lasted up to at least 2 h after administration. In a recent study measuring hippocampal neurotransmitter efflux in rats, in vivo microdialysis was paired with an ORT paradigm [27]. While glutamate efflux did not increase above baseline levels during exposure of familiar objects, it was significantly increased when a novel object was shown 2 h later in the second ORT trial. This strongly suggests that hippocampal glutamate is involved in memory formation processes. These results might explain the discrepancy in active MLA doses of our ORT (0.1 mg/kg) and microdialysis studies (1 mg/kg). Considering the increased glutamate efflux during exposure to novel objects alone, the combination of MLA administration plus novel object exposure would probably lead to additive glutamate effects. This could explain the difference in active doses. We hypothesize that combining microdialysis with an ORT paradigm could have led to lower active MLA doses, meaning that glutamate efflux is more optimal with lower MLA doses when combined with novelty exposure.

The electrophysiological studies conducted on isolated hippocampal slices revealed that low concentrations of MLA (30 nM, 100 nM) induced a stronger LTP when administered before a weak tetanus. Conversely, perfusion of the slices with low concentrations of MLA after LTP induction or high concentrations before tetanus did not alter potentiation.

At low doses, MLA improved the memory acquisition processes of rats in the ORT natural forgetting (24 h retention interval) paradigm (optimal dose range: 0.003–0.1 mg/kg, i.p.). On the contrary, when the doses were increased (1.0 mg/kg i.p.), and as expected from our current knowledge, MLA caused a memory deficit in the 1 h retention interval ORT, an interval that normally leads to good memory performance of rats (see for example [26]). The observed memory deficit upon 1.0 mg/kg i.p. MLA administration is in accordance with earlier studies using relatively high doses of MLA to induce cognitive impairments. Intraperitoneal (0.3 mg/kg) or intracerebrovascular (10 µg)

administration of MLA both prevented procognitive effects by the α7nAChR partial agonist EVP-6124 (encenicline) [26]. These doses are comparable to cognition-impairing doses used for local MLA infusion into the dorsal hippocampus of male mice (1–30 μg/side) [53] or into the dorsal hippocampus or basolateral amygdala of female rats (6.75 μg/side) [54,55]. The memory-enhancing doses (i.p. administration) used in the current study are 10–330 times lower than the memory-impairing doses. Hence, future studies have to reveal whether lower doses using different administration routes as used in other studies may also lead to α7nAChR potentiation rather than inhibition.

ORT experiments to investigate the effects of the α7nAChR antagonist specifically on memory consolidation processes were performed as well by administering MLA i.p. after T1 [33]. The results showed that MLA doses optimal for memory acquisition processes had no effect on memory consolidation processes. This is in line with the electrophysiological LTP studies, where it was shown that a low dose of MLA before, but not after LTP induction led to a longer-lasting potentiation. Therefore, both these behavioral and electrophysiological studies suggest that α7nAChR antagonism has effects only on the acquisition-like memory processes.

In the ORT memory consolidation study, an effect of MLA was found on the exploratory behavior of the rats in T2. Specifically, the exploration behavior of the rats in the 0.1 mg/kg condition was lower than in the vehicle or 0.03 mg/kg MLA condition. Since T2 was almost 24 h after the MLA injection and considering the short half-life (± 37 min) and fast peak brain concentration (30 min) of MLA in rats [40], the observed difference in exploratory behavior was unlikely to be related to the tested MLA concentration. The mean exploration times of the animals were always sufficient (> 20 s, data not shown) to draw reliable conclusions and the discrimination measure (d2) used corrects for possible differences in exploratory activity [39]. MLA is highly specific for α7nAChRs with a differential affinity of a 100-fold or more versus other receptors such as α3β2, α4β2 and muscle nAChRs [21–25]. Since MLA at low concentrations antagonizes the effects of highly selective α7nAChR agonists [26,56], no effects of MLA on other ligand-gated ion channels or G-protein coupled receptors are expected. Moreover, given the low active doses, the effects reported above are unlikely to be caused by heteromeric receptors and are therefore attributed to α7 containing receptors.

Since memantine, a NMDA receptor antagonist clinically used to treat Alzheimer's disease, is also a potent α7nAChR antagonist [57], part of its procognitive effect may be due to actions on α7nAChRs. Active doses ranges in the brain may primarily affect α7nAChRs rather than NMDA receptors. Similarly to our observations with MLA, at low concentrations memantine may bind no more than two bindings sites per α7nAChR which may lead to a potentiating rather than an inhibitory effect. Whether memantine act on α7nAChRs in vivo and what its effect is on receptor functionality at particular doses remains to be elucidated.

5. Conclusions

Low concentrations of the selective α7nAChR antagonist MLA potentiate receptor activity leading to an increased Ca^{2+} influx. The degree by which MLA inhibits or potentiates receptor responses seems in both cases to be dependent on the duration of MLA incubation. The exact mechanisms underlying these different actions remain to be unraveled. Increased receptor activity by MLA and associated Ca^{2+} influx is consistent with the observed MLA-induced increase in glutamate efflux. Increased glutamate signaling could explain the observation that low dose MLA administration converted *E*-LTP to L-LTP before, but not after tetanus in electrophysiological recordings. Accordingly, low doses of MLA specifically improve memory acquisition processes, but not memory consolidation processes. These findings show that a selective α7nAChR antagonist, in low doses, can improve memory. Finally, while the main focus of the α7nAChR as a target for cognition enhancement

has traditionally involved agonists and positive modulators, antagonists at appropriate doses may also prove to be a valuable tool for cognition enhancement in for instance schizophrenia or Alzheimer's disease.

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Declaration of competing interests

The authors declare that they have no competing interests.

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