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Alpha 7 Nicotinic Receptor Agonist and Positive Allosteric Modulators Improved Social and Molecular Deficits of MK-801 Model of Schizophrenia in Rats

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Abstract

Schizophrenia is a common psychiatric disease that cannot be fully treated with current antipsychotic drugs. It has shown that glutamatergic NMDA receptor antagonists such as MK-801 cause schizophrenia-like phenotype in rodents. Recent studies indicated that α7 nicotinic acetylcholine receptor (nAChR) deficits contribute to schizophrenia. Enhancing its activity with agonist or positive allosteric modulators (PAMs) may be a valuable approach for treatment. The certain intracellular pathways such as Akt/Glycogen synthase kinase 3 beta (GSK-3β) and phosphodiesterase-4 (PDE-4)/cAMP are associated with the pathogenesis of schizophrenia. In this study, we examined the effect of α7 nAChR agonists and PAMs on the behavioral and molecular phenotype of schizophrenia in the subchronic MK-801 administered rats. Social interaction, the levels of α7 nAChR, and related intracellular pathways (cAMP, PDE4A, PDE4D, p-Akt/Akt, p-GSK-3β/GSK-3β) were measured by behavioral or ELISA and western blot tests. Subchronic MK-801 administration decreased the following behaviors and increased the avoiding behaviors. However, only α7 nAChR agonist (A-582941) increased the following behavior while α7 nAChR agonist, PAMs (CCMI and PNU-120596), and clozapine decreased the avoiding behavior compared to MK-801. For molecular parameters, MK-801 administration decreased the α7 nAChR, p-Akt/Akt, p-GSK-3β/GSK-3β expressions, and cAMP levels while it increased PDE4A, PDE4D expressions in the prefrontal cortex. Besides, MK-801 decreased the α7 nAChR, p-GSK-3β/GSK-3β expressions in the hippocampus. We found clozapine, α7 nAChR agonists, and PAMs reversed the molecular deficits induced by MK-801. Herein, we showed that prefrontal cortex is more sensitive to the devastating effects of subchronic MK-801 administration, especially for PDE4, in rats. In addition to clozapine, α7 nAChR agonists and PAMs found to be beneficial on both social and molecular deficits induced by MK-801 in rats. We suggested that α7 nAChR agonists and PAMs might be valuable approaches to treat negative symptoms of schizophrenia when unmet needs and current limitations considered in this pathology.

Keywords: A-582941; CCMI; PNU-120596; MK-801; α7 nAChR; Schizophrenia

1. Introduction

Schizophrenia is a severe psychiatric disease with approximately 1% prevalence. The symptoms of schizophrenia consist of three main groups as positive (hallucination, delusion, etc.), negative (lack of socialization and motivation, etc.) and cognitive (learning and attention deficits) symptoms. In current medication, typical (first generation) and atypical (second generation) antipsychotics are used to treat schizophrenia patients [1]. However, two certain large scale effectiveness studies with no sponsorship, CATIE (Clinical Antipsychotic Trials of Intervention Effectiveness) and CUtLASS (Cost Utility of the Latest Antipsychotic Drugs in Schizophrenia Study) have found no superiority of atypical antipsychotic drugs on typicals for the treatment of negative and cognitive symptoms. In contrast, atypicals had advantages for certain side effects such as extrapyramidal system [2]. Besides, the long term usage of typical antipsychotics causes severe extrapyramidal side effects such as akathisia and tardive dyskinesia while atypicals reveal agranulocytosis and metabolic syndrome in patients. For these reasons, the novel approaches to treat schizophrenia will be very valuable for patients.

Glutamatergic hypoactivity is one of the most accepted hypotheses about the pathophysiology of schizophrenia. It has mentioned that a decrease of glutamatergic N-Methyl-D-Aspartate (NMDA) receptor function in the cortical-brainstem pathway causes symptoms of schizophrenia. Previous studies have well demonstrated that the administration of NMDA receptor antagonists such as MK-801 and Phencyclidine reveals schizophrenia-like behavioral and neurobiological changes in rodents [3]. Sam's Dodd et al. (1999) showed that NMDA receptor antagonism caused social deficits in rats, and the model had a face and predictive validity in the social interaction test [4]. It has shown that subchronic injections (bi-daily for seven days) of NMDA receptor antagonists with a seven day washout period are well characterized with schizophrenia in rats [5].

It has been known that disruption of the balance between cholinergic and dopaminergic systems contribute to the pathophysiology of schizophrenia [6,7]. Recent studies have indicated that nicotinic acetylcholine receptor (nAChR), especially α7 subtype dysfunction, is closely related to the sensorimotor deficits in schizophrenia [6]. In addition to this, the higher prevalence of smoking in schizophrenia patients was associated with their self-treatment desire. Therefore, enhancing the function of α7 nAChR in the brain became one of the promising targets in the treatment of schizophrenia. The first attempt to solve this problem about α7 nAChR was the development of specific α7 nAChR full and partial agonists. Studies showed that α7 nAChR agonists such as A-582941 improved not only cognitive symptoms of schizophrenia but also reversed GABAergic deficits, including the decrease in the parvalbumin and GAD67 expressions in the brain of rats [8,9]. Moreover, it has been indicated that α7 nAChR agonists increase acetylcholine release from the presynaptic neurons. However, it has an "inverse U" shape dose-response curve because of the low efficacy at the low dose and rapid desensitization on the higher dose of agonists. On the contrary to our pharmacological knowledge, certain studies showed that α7 nAChR agonists but not PAMs cause a receptor up-regulation instead of downregulation [9]. Therefore, the effects of α7 nAChR agonists should be considered as the sum of the factors mentioned above. α7 nAChR positive allosteric modulators (PAMs) were developed as an alternative approach to overcome the disadvantages of agonists, such as rapid desensitization. PAMs are the molecules that not induce a response when it binds to the receptor, but it enhances the effect of natural and exogenous agonists of α7 nAChR. α7 nAChR agonists reveal their effects via orthostatic binding sites, while PAMs mediate its effects via allosteric binding sites of receptors. PAMs are divided into two groups as type I and type II according to the pharmacological profile in the presence of endogenous agonists. Type I PAMs such as CCMI increase the ion permeability of receptor and increase agonist-evoked peak amplitude without changing the open time of the ion channel while Type II PAMs such as PNU-120596 increase both ion permeability and open time of receptor in electrophysiological studies. Another critical difference between the two classes is that type I PAMs do not alter the natural desensitization kinetics of the receptor, whereas type II PAMs slow the desensitization of receptors and re-sensitize them [10]. Previous studies have shown that a single dose administration of A-582941 revealed procognitive

effects in the healthy rats and antipsychotic-like effects in the rat model of schizophrenia [11,12]. To our current knowledge, there is no study investigating the effect of chronic administration of A-582941 in the schizophrenia model of rats. Similarly, it has been reported that CCMI, α7 nAChR type I PAM, and PNU-120596, α7 nAChR type II PAM, had procognitive effects and reversed schizophrenia-like behaviors when administered in a single injection [13-15]. However, the neurobiological effects of A-582941, CCMI, and PNU-120596 on the social deficits of schizophrenia have not fully understood yet.

Recent studies have shown that the intracellular Akt/Glycogen synthase kinase 3 beta (GSK-3β) signaling pathway might play a crucial role in the pathophysiology of schizophrenia. It has been proved that deactivation (phosphorylation) of the GSK-3β enzyme was decreased due to decreased phosphorylation (activation) of its inhibitor up-stream regulatory protein Akt in the brain tissues of schizophrenia patients and related animal models. Besides, the limited number of studies indicated that current antipsychotic drugs increased Akt and GSK-3β phosphorylation in the hippocampus and prefrontal cortex of rodents [16]. One of the recent approaches to the novel treatments for schizophrenia is the inhibition of the increased phosphodiesterase (PDE) activity in the brain. Primarily PDE4 and PDE10 are held responsible for schizophrenia, even though there are 21 different PDE encoding genes for the 11 different PDE subclasses (PDE1-11) of these enzymes. The PDE enzyme subtypes have a different affinity to the cyclic adenosine monophosphate (cAMP) and cyclic guanidine monophosphate (cGMP) proteins, which are the two of the principal seconder messengers in the cell. PDE4 enzymes which are subclassified as PDE4A, PDE4B, and PDE4D due to their encoding genes are the unique subtypes showing only cAMP but not cGMP affinity in cells. The increased PDE4 enzyme activity causes over inhibition of the cAMP via its phosphorylation in schizophrenia, and it has been suggested that increasing the cAMP activity with PDE4 inhibitors might be a valuable approach to treat schizophrenia [17]. For these reasons, PDE4A, PDE4D, p-Akt/Akt, p-GSK-3β/GSK-3β protein expressions, and cAMP protein levels were evaluated to determine its relationship with α7 nAChR in this study.

In the light of the data summarized above, the effects of α7 nAChR partial agonist (A-582941), type I PAM (CCMI) and type II PAM (PNU-120596) were aimed to investigate on the social deficits and underlying molecular mechanisms in subchronic MK-801 model of schizophrenia in rats. We comparatively investigated the effects of these ligands with clozapine, a gold standard atypical antipsychotic drug, and proved the revertive effect of NMDA receptor antagonists-induced social deficits [4], on both behavioral and molecular alterations of MK-801 model. For this aim, the intracellular levels of cAMP, PDE4, Akt, and GSK-3β were measured to understand the intracellular mechanism of therapeutic effects of α7 nAChR ligands on social deficits of schizophrenia.

2. Experimental Procedures

2.1. Animals and housing

All experiments were performed under the Regulation of Animal Research Ethics Committee in Turkey (6 July 2006, Number 26220). The Erciyes University Animal Research Ethics Committee approved this study (protocol number 16/019). All animals were housed for 14 days before the experiments to decrease the new environmental stress of the laboratory. Male Wistar Hannover rats (8-12 weeks and 180-250g) were used in this study. The room temperature (22±1 °C) and dark/light cycle (12/12) were stable during the experiments. Rats were fed ad libitum in this study. Animals were divided into six groups as the following: vehicle (n=8), MK-801 (n=8), MK-801+Clozapine (n=8), MK-801+A-582941 (n=8), MK-801+CCMI (n=8), MK-801+PNU-120596 (n=8) (n=4 per groups for ELISA and western blotting).

2.2. Drugs

(+)-MK-801 hydrogen maleate (Cayman, USA) was dissolved in a trace amount of dimethyl sulfoxide (MERK, Germany) and freshly diluted with saline in every injection day. Clozapine (Leponex©, Novartis), A-582941 (Santa Cruz, USA), CCMI (Santa Cruz, USA), PNU-120596 (Selleckchem, USA) were dissolved into dimethyl sulfoxide and diluted with saline. The final concentration of DMSO was 10%. Drug solutions were injected at 0.1 ml / 100 g to rats, and the vehicle group took an equal volume of diluted dimethyl sulfoxide.

2.3. Experimental Desing and Drug Treatments

After the 14 days of the habituation period, MK-801 (0.2 mg/kg) was bidaily (07:00 am and 07:00 pm) administered for seven days via the intraperitoneal route in rats. Then, a seven days washout period was conducted to establish chronic schizophrenia model and ten days daily treatment session of clozapine (5 mg/kg), A-582941 (1 mg/kg), CCMI (1 mg/kg) and PNU-120596 (3 mg/kg) were intraperitoneally administered to related groups. In the 10th day of treatments, social interaction test was performed either 30 or 45 min (for A-582941) after the last injections depending on the pharmacokinetic profile of the drugs. The specific doses of the α7 nAChR ligands were determined according to our previous studies observing their procognitive effects on the same doses of ligands. Certain studies also provided benefits to determine the doses of α7 nAChR (10). For the molecular analyses, the rats were decapitated, and brain tissues were dissected 24 hours after the last treatments. Hippocampal and prefrontal tissues of the rats used in the social interaction test were used in Enzyme-Linked ImmunoSorbent Assay (ELISA) and western blot analyses for evaluating protein expressions.

2.4. Social Interaction (SI)

SI was conducted in a black plexiglass chamber (50x50x30 cm) at semi-light conditions. The SI protocol consisted of two different periods as the habituation (first day) and test (second day) protocols. In habituation protocol, all animals in the same cage allowed to move freely in the test apparatus for 60 minutes for decreasing the new environmental stress of test protocol. In the SI test protocol, two unfamiliar rats placed into the same chamber and allowed to spend time for 10 min freely. In this step, to minimize and standardize the effects of the other rat's behavior, the second rat was selected by a healthy rat, and its behavior did not score in the test. It has meant that all rats' behaviors used in this study were measured against a healthy rat, which did not use in this study. The social behaviors of rats are affected by its cagemate`s sociality. It was aimed to standardize the social behaviors of second rats in this study. During this period, spending time following, avoiding, and sniffing behaviors were scored for each rat. The following behavior was considered a clear pursuit of rat A to rat B when rat A stands or goes opposite directions to rat B. The avoiding behavior was considered a turning rat A" back to the rat B when they came face to face in the chamber. Sniffing behavior was considered as a direct sniffing behavior showing its affinity to the other one as an indicator of identifying its demand but not climbing behavior. Total interaction was calculated for each rat according to the following formula: Times for

the Following + Sniffing – Avoiding behaviors. In each test, one of the rats was temporarily colored with odorless ink for discrimination of rats. The chamber was cleaned with ethanol (70%) after each test [18]. All the behaviors were scored by an experimenter who has done the experiments and treatment blind researcher after all descriptive data of treatments (video names, treatment names, etc.) were removed from videos, and the mean scores of two researchers were used in our statistical analyses.

2.5. Enzyme-Linked ImmunoSorbent Assay (ELISA)

Tissues were homogenized in pH 7.4 phosphate-buffered saline containing a protease inhibitor cocktail. The homogenate was centrifuged at 10 000 rpm for 10 min to remove debris and supernatant used for the analysis of total protein content. The total protein amounts were determined by the Pierce bicinchoninic acid (BCA) assay kit (Thermo Fisher Scientific, Waltham, MA USA) and normalized using bovine serum albumin as standard. After the determination of total protein amounts of the tissues, the equal amounts of them used for the ELISA test. The cAMP levels in the prefrontal cortex and hippocampus were measured using a sandwich ELISA using the commercially available kit (Cayman Chemical Company, Ann Arbor, MI, USA.) following the manufacturer's instructions.

2.6. Western Blotting

The prefrontal cortex and hippocampus tissue samples were homogenized in pH 7.4 phosphate-buffered saline containing a protease inhibitor cocktail. The homogenate was centrifuged at 10 000 rpm for 10 min to remove debris. Supernatant used for the analysis of total protein content with Pierce BCA assay kit (Thermo Fisher Scientific, Waltham, MA USA). Equal amounts (40 μg/mL) of protein are denatured in SDS, electrophoresed on 8-12 % SDS-PAGE and followed by transferring onto PVDF membrane (Bio-Rad, Hercules, CA, USA) and blocked with 5 % nonfat dry milk in TBST. Membranes were incubated with appropriate primary antibody anti-α7 nAChR (1:1000; Abcam, Cambridge, UK), anti-PDE4A (1:1000; MyBioSource, CA, USA), anti-PDE4D (1:1000; MyBioSource, CA, USA), anti-Akt (1:1000; Cell Signaling Technology, MA, USA), anti-p-Akt (1:1000; Cell Signaling Technology, MA, USA), anti-p-GSK-3β (1:1000; Cell Signaling Technology, MA, USA), anti-GSK-3β (1:1000) (Cell Signaling Technology, MA, USA) and GAPDH (1:1000; Cell Signaling Technology, MA, USA) at 4 °C for overnight and then incubated with horseradish peroxide-conjugated secondary antibody (anti-IgG HRP) room temperature for 2 h. Specific bands were visualized by chemiluminescence using the Super Signal West Pico (Pierce, IL, USA).

2.7. Statistical Analysis

Statistical analyses were performed by Graph Pad Prism 6.0 software. One way analyses of variance (ANOVA) were performed for molecular analyses and social interaction tests. Dunnett's post hoc test was used for the comparison of groups. Data were presented as mean ± standard error of the mean (SEM), and $p<0.05$ was accepted as a value of significance. Full statistical details of the ANOVA test, including F= (DFn, DFd), were presented in the results sections.

3. Results

3.1. Social behaviors of MK-801 and treatment groups

The rats in the MK-801 group showed significantly decreased ($p<0.05$; $F(5, 42) = 8.099$) following behavior and increased ($p<0.001$; $F(5, 42) = 13.84$) avoiding behavior compared to the vehicle group. There are no significant changes among all groups for sniffing behavior and total interactions, even though there were small fluctuations in them ($F(5, 42) = 3.187$ for sniffing and $F(5, 42) = 2.850$ for total interactions). Clozapine ($p<0.01$), A-582941 ($p<0.001$), CCMI ($p<0.01$) and PNU-120596 ($p<0.001$) treatments reduced ($F(5, 42) = 13.84$) avoiding behavior whereas only A-582941 increased ($p<0.001$; ($F(5, 42) = 8.099$) following behavior compared to MK-801 group (Fig. 1).

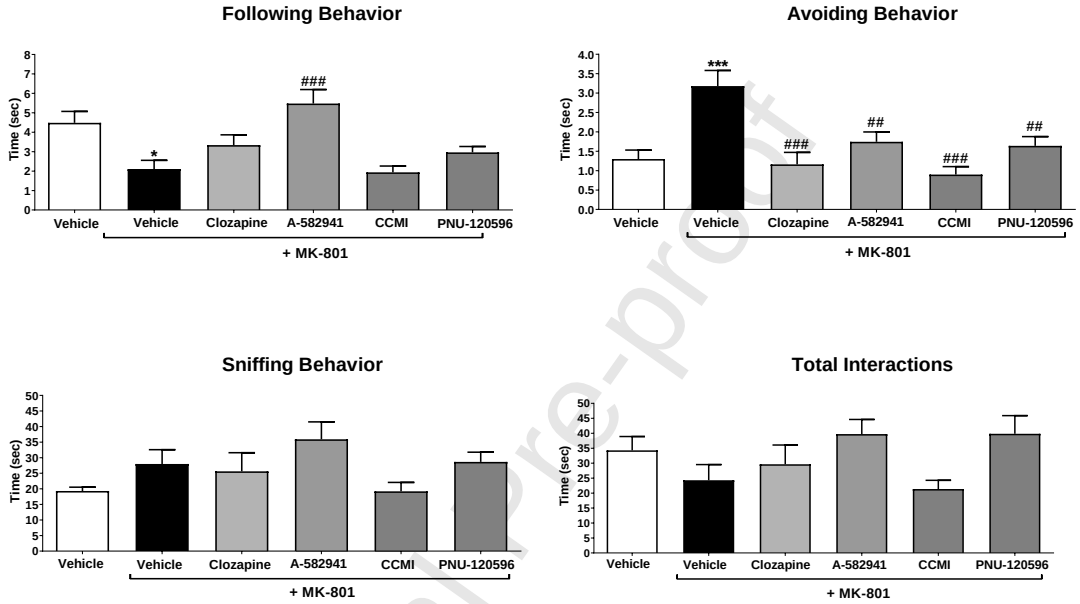

Fig. 1. The effects of clozapine, A-582941, CCMI, and PNU-120596 treatments on MK-801 induced social deficits. Data were presented as mean ± SEM, and one-way ANOVA followed by Dunnett's post hoc test was used for statistical analysis. Symbols mean the following: *: $p<0.05$ and ***: $p<0.001$ compared to vehicle group and ##: $p<0.01$ and ###: $p<0.001$ compared to MK-801 group.

3.2. α7 nAChR receptor protein expression in prefrontal cortex and hippocampus of rats

In both prefrontal cortex and hippocampus, MK-801 administered rats had markedly lower α7 nAChR protein expressions than the vehicle group ($p<0.05$). Clozapine ($p<0.05$, $p<0.01$), A-582941 ($p<0.05$, $p<0.01$), CCMI ($p<0.001$), and PNU-120596 ($p<0.01$) raised the level of α7 nAChR protein expressions compared with MK-801 in prefrontal cortex and hippocampus ($F(5, 18) = 29.06$ for prefrontal cortex and $F(5, 18) = 22.34$ for hippocampus; Fig. 2).

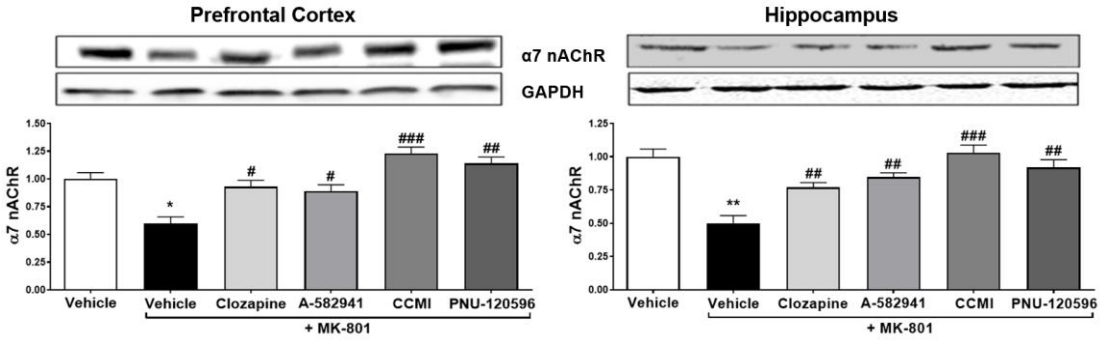

Fig. 2. Effects of clozapine, A-582941, CCMI, and PNU-120596 on α7 nAChR protein expression in the prefrontal cortex and hippocampus of MK-801 treated rats. Data were presented as mean ± SEM, and one-way ANOVA followed by Dunnett's post hoc test was used for statistical analysis. *: $p<0.05$, **: $p<0.01$ compared to vehicle and #: $p<0.05$, ##: $p<0.001$, ###: $p<0.001$ compared to MK-801 group.

3.3. cAMP levels in prefrontal cortex and hippocampus of rats

In the prefrontal cortex, MK-801 injection leads to decrease ($p<0.01$) in cAMP levels compared to vehicle when clozapine ($p<0.01$), A-582941 ($p<0.01$), CCMI ($p<0.001$), and PNU-120596 ($p<0.05$) elevated cAMP levels compared to MK-801 groups ($F(5, 18) = 36.91$). However, there was no significant difference among all groups in the hippocampus ($F(5, 18) = 3.92$; Fig. 3).

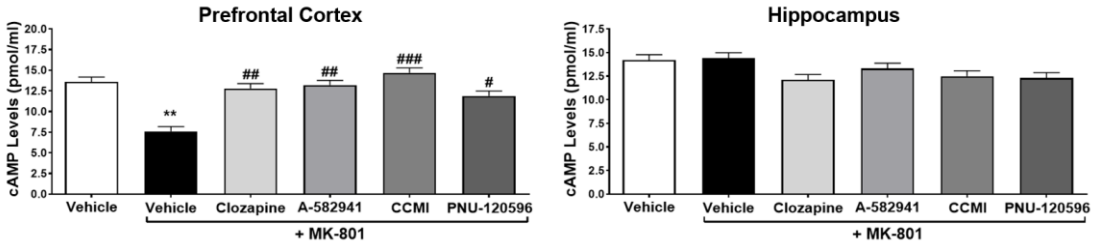

Fig. 3. Effects of clozapine, A-582941, CCMI, and PNU-120596 on cAMP levels in the prefrontal cortex and hippocampus of MK-801 treated rats. Data were presented as mean ± SEM and one-way ANOVA followed by Dunnett's post hoc test was used for statistical analysis **: $p<0.01$ compared to vehicle and #: $p<0.05$, ##: $p<0.001$, ###: $p<0.001$ compared to MK-801 group.

3.4. PDE4A and PDE4D protein expressions in prefrontal cortex and hippocampus of rats

The expressions of PDE4A and PDE4D were found to increased ($p<0.001$) in the prefrontal cortex of MK-801 administered rats compared to the vehicle while none of the PDE4 subtypes was significantly changed by MK-801 in the hippocampus of rats. Clozapine, A-582941, CCMI, and PNU-120596 reduced PDE4A ($p<0.001$, $p<0.05$, $p<0.001$ and, $p<0.001$; $F(5, 18) = 41.54$) and PDE4D ($p<0.05$, $p<0.01$, $p<0.001$ and, $p<0.001$; $F(5, 18) = 37.22$) protein expressions in the prefrontal cortex compared to MK-801. In the hippocampus, only CCMI treatment decreased ($p<0.05$; $F(5, 18) = 8.731$) the PDE4A protein expression compared to MK-801 group even though MK-801 did not significantly change the protein levels in this region whereas there was no significant alteration ($F(5, 18) = 2.127$) for PDE4D protein expressions (Fig. 4).

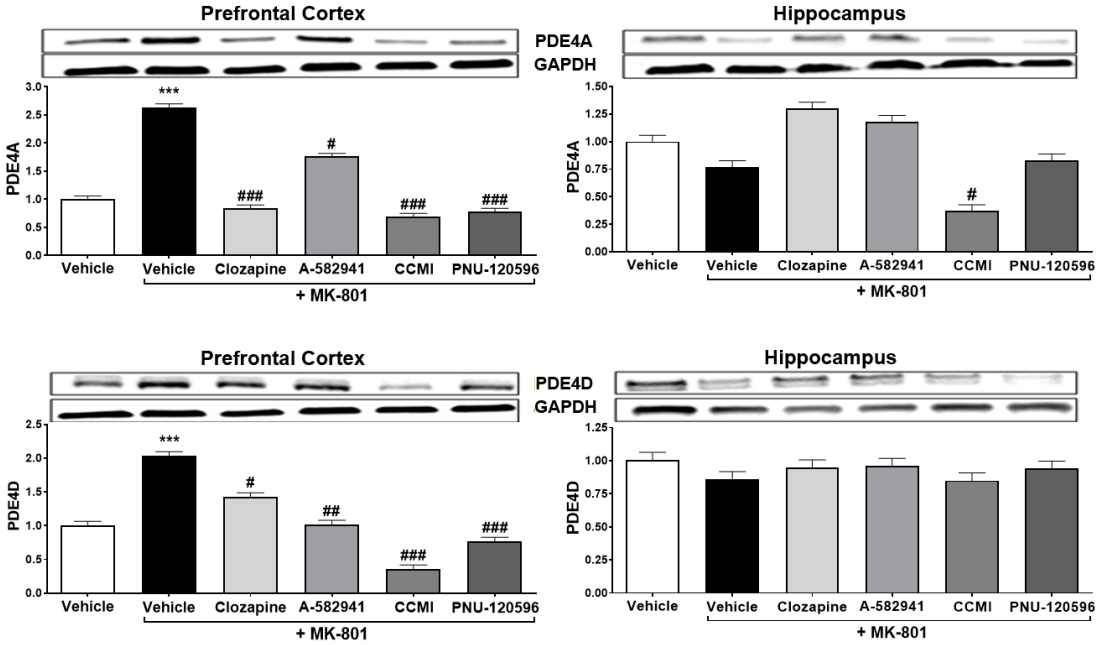

Fig. 4. Effects of clozapine, A-582941, CCMI, and PNU-120596 on the PDE4A and PDE4D protein expressions in the prefrontal cortex and hippocampus of MK-801 treated rats. Data were presented as mean ± SEM, and one-way ANOVA followed by Dunnett's post hoc test was used for statistical analysis. ***: p<0.001 compared to vehicle and #: p<0.05, ##: p<0.001, ###: p<0.001 compared to MK-801 group.

3.5. The ratio of p-Akt/Akt and p-GSK-3β/GSK-3β protein levels in prefrontal cortex and hippocampus of rats

The ratio of p-Akt to Akt protein expression was examined to investigate the Akt activity by western blot test. MK-801 administration down-regulated the p-Akt /Akt ratio compared to vehicle in prefrontal cortex ($F(5, 18) = 33.88$) but not hippocampus ($F(5, 18) = 7.798$). In prefrontal cortex, clozapine ($p<0.05$), A-582941 ($p<0.001$), CCMI ($p<0.001$), and PNU-120596 ($p<0.05$) significantly elevated the ratio compared to MK-801 group ($F(5, 18) = 33.88$). In the hippocampus, only A-582941 administration markedly increased ($p<0.05$) the p-Akt /Akt ratio compared to the MK-801 group ($F(5, 18) = 7.798$; Fig. 5).

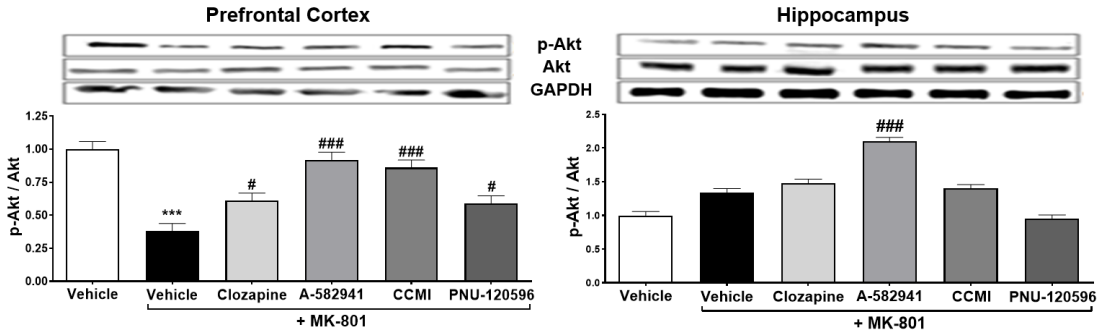

Fig. 5. Effects of clozapine, A-582941, CCMI, and PNU-120596 on p-Akt/Akt protein expressions in the prefrontal cortex and hippocampus of MK-801 treated rats. Data were presented as mean ± SEM, and one-way ANOVA followed by Dunnett's post hoc test was used for statistical analysis. ***: p<0.001 compared to vehicle and #: p<0.05, ###: p<0.001 compared to MK-801 group.

The ratio of p-GSK-3β (inactive) to GSK-3β (active) protein expressions was used to evaluate the GSK-3β activity in this study. MK-801 administration markedly decreased ($p<0.001$) the p-GSK-

3β/GSK-3β ratio compared to the vehicle in the prefrontal cortex and hippocampus of rats. The clozapine ($p<0.01$), A-582941 ($p<0.01$, $p<0.001$), CCMI ($p<0.01$, $p<0.001$), and PNU-120596 ($p<0.001$) treatments significantly increased the ratio compared to MK-801 in prefrontal cortex ($F(5, 18) = 60.67$) and hippocampus ($F(5, 18) = 76.21$) of rats (Fig. 6).

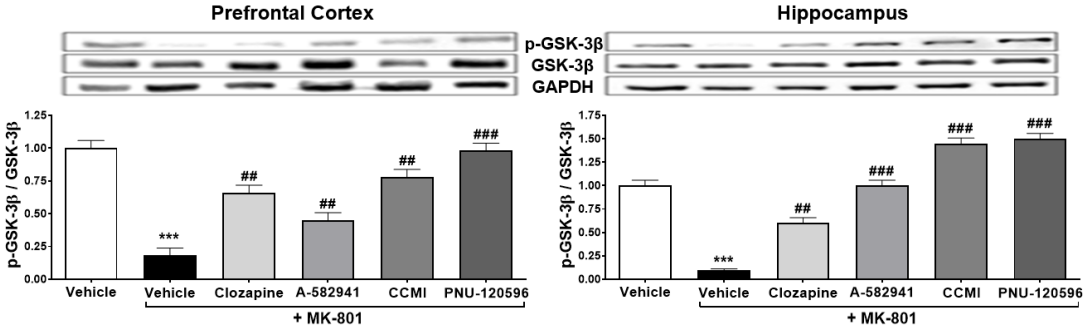

Fig. 6. Effects of clozapine, A-582941, CCMI, and PNU-120596 on p-GSK-3β/GSK-3β protein expression in the prefrontal cortex and hippocampus of MK-801 treated rats. Data were presented as mean ± SEM, and one-way ANOVA followed by Dunnett's post hoc test was used for statistical analysis. ***: $p<0.001$ compared to vehicle and ##: $p<0.01$, ###: $p<0.001$ compared to MK-801 group.

4. Discussion

In this study, we aimed to evaluate the effects of α7 nAChR agonist (A-582941) and PAMs (CCMI and PNU-120596) on the social deficits of schizophrenia and possible underlying molecular mechanisms in a well-validated rat model. Our results indicated that the subchronic MK-801 administration caused social withdrawal-like behaviors such as less interaction and more avoiding behaviors in rats. It has been shown that intracellular cAMP/PDE4 and Akt/ GSK-3β pathways disruptions, especially in the prefrontal cortex, accompanied to social deficits in rats. Also, the protein expression of α7 nAChR was repressed by MK-801 in the prefrontal cortex and hippocampus of rats. There is not a precise biological explanation for the social deficits of schizophrenia, even though the common opinion is that prefrontal cortical dysfunction or damages are responsible for the negative symptoms of schizophrenia. The limited number of studies showed that an increase of the α7 nAChR function by an agonist or PAMs improved the social deficits of schizophrenia in an acute ketamine model of rats [12]. There is no study on the effects of α7 nAChR agonists and PAMs on the possible underlying molecular mechanisms of the social deficits of schizophrenia in rats. Herein, we demonstrated that α7 nAChR agonists and PAMs might be beneficial effects on the social deficits of schizophrenia in rats. Moreover, besides the decreased α7 nAChR expressions, these ligands also improved the specific intracellular deficits, including decreased cAMP levels, Akt and GSK-3β phosphorylations, and increased PDE4A, PDE4D expressions in prefrontal cortex and hippocampus of rats.

Based on the behavioral studies performed to date, α7 nAChR agonist approaches are very promising and still entirely new. Although some clinical data demonstrate that α7 nAChR orthosteric agonists exert therapeutical effects, their clinical efficacy in schizophrenic patients remains uncertain [19-22]. As an alternative approach to improve the function of α7 nAChRs, the usage of its PAMs seems to be one of the most promising novel approaches to treat the several symptoms of schizophrenia [23-25]. Herein, we have shown that α7 nAChR agonists had partial superiority because of additional improvement of the following behaviors compared to α7 nAChR PAMs. This difference is not a situation that can be explained very easily. It is known that one of the principal differences between agonists and PAMs is that agonists alone can create a response at the receptor, while PAMs can only increase the existing response of agonists. In addition, it has been shown that α7 nAChR agonists caused endogenous acetylcholine release from the presynaptic cells [9]. Although the amounts of acetylcholine, the nAChR natural agonist, in the brain tissues related to social behaviors have not been studied in our study, our data made us think that the agonist might better compensate a possible reduction in the amount of endogenous agonist because the activity of PAMs was limited in the lack of an agonist. Relative to the literature on α7nAChR ligands, there is still much to be done to conclusively determine the potential of α7 nAChR PAMs for the treatment of schizophrenia and to shed light on the molecular mechanisms underlying the intracellular trafficking related to these beneficial effects.

Our knowledge about the efficacy of α7 nAChR agonists and PAMs on principal intracellular second messengers systems such as the cAMP system in the brain is limited. For these reasons, we have designated to elucidate whether these ligands were able to overcome the disruptive effects of subchronic MK-801 on the main intracellular trafficking pathways including cAMP and its upstream regulators PDE4A and PDE4D pathway in this study. It has been indicated that PNU-120596 increased the phosphorylation of cAMP response element-binding protein (CREB), a well-recognized biochemical process implicated in learning and memory [26]. Our present study is the first known report revealing the relationship between the α7 nAChR ligands, PDE4A, and PDE4D expressions and intracellular cAMP levels in the hippocampus and prefrontal cortex in subchronic MK-801 model of schizophrenia. We showed that MK801 administration significantly increased the PDE4A and PDE4D expressions in the prefrontal cortex, which was reversed by α7 nAChR agonist, PAMs, and clozapine. However, these effects did not achieve any significance in the hippocampus except CCMI for PDE4A. The cAMP levels significantly decreased by subchronic MK-801 administration in the prefrontal cortex, whereas it did not change in the hippocampus of rats. All the α7 nAChR ligands tested in this study increased α7 nAChR protein expressions in both the hippocampus and prefrontal cortex. Our

results also showed an α7 nAChR down-regulation induced by MK-801 and the revertive effects of its all ligands and clozapine in the prefrontal cortex and hippocampus of rats. Previous reports indicated that α7 nAChR agonists but not PAMs induced the receptor up-regulation in cells [27]. We can hypothesize this mechanism as a cellular demand to protect its anionic-cationic balance and re-excitability even though there is a limited number of studies showing their effects on α7 nAChR expressions. We have known that especially full and also partial α7 nAChR agonists cause rapid receptor desensitization due to preventing the excessive positive ion entrance to the cells. However, the cells need the new receptors for their next physiological stimulation and up-regulate these receptors, probably for this reason. So, we have thought that PAMs do not cause receptor up-regulation because they do not desensitize α7 nAChR receptors in the physiological circumstances. Moreover, it has been shown that type I PAMs did not influence the agonist-induced up-regulation while type II PAMs inhibited it [27]. It has been thought that type I PAMs do not inhibit the agonist-induced receptor up-regulation because of no effects on desensitization kinetic while type II PAM inhibits it because they also re-sensitize the α7 nAChR and do not need any new receptor expressions. Herein, our results based on the α7 nAChR expressions in the brain were substantially different from the previous report. In our study, we reported the α7 nAChR up-regulation with not only agonists but also PAMs and even clozapine treatments in subchronic MK-801 administered rats. All the hypotheses and effects mentioned above were valid in the presence of α7 nAChR ligands in the medium without any different molecules and disease models while the effects of ligands used in our study were investigated on the α7 nAChR expressions already down-regulated by MK-801 in the rat brains. For this reason, all the up-regulative effects of α7 nAChR ligands and also clozapine might be depending on their revertive effects of MK-801 induced damages in the brain.

The intracellular GSK-3β signaling pathway is also an outstanding molecular target for schizophrenia. It has been hypothesized that the decrease in the phosphorylation of Akt causes an increase in the GSK-3β activity because of its hypo-phosphorylation by Akt. It has also been indicated that the hyperactivation of GSK-3β induces neurodegeneration in nerve cells via different molecular mechanisms [28]. Furthermore, no cytotoxic effects were detected when type I or type II PAMs have applied alone or combined with a selective α7 nAChR agonist as assessed by changes in adenylate kinase release or mitochondrial dehydrogenase activity in both PC12 cells and cortical neurons [30]. In our study, MK-801 decreased the phosphorylation of Akt and GSK-3β in the prefrontal cortex and hippocampus of rats. In the prefrontal cortex, α7 nAChR agonist, Type I and Type II PAMs increased the phosphorylation of Akt and GSK-3β and therefore reduced the activity of GSK-3β. In addition to these, MK-801 increased the GSK-3β activity (p-GSK-3β/GSK-3β) but not Akt activity (p-Akt/Akt ratio) in the hippocampus of rats. α7 nAChR agonist and PAMs increased the phosphorylation of GSK-3β and therefore reduced the activity of GSK-3β in the hippocampus of rats. In line with our study, previous reports have demonstrated that both the acute and continuous (2 weeks) infusion administrations of A-582941 increased the phosphorylation of the GSK-3β in the mouse cingulate cortex and hippocampus [29]. Besides, it has also been shown that full agonist (ABT-107) increased the GSK-3β phosphorylation in the rat cortex [30]. Moreover, it has been indicated that α7 nAChR agonists increased the Akt activity in cell culture [31]. In the sum of these, it might be revealed that the α7 nAChR ligands inhibit the GSK-3β activity probably using the Akt pathway in the prefrontal cortex and but other intracellular pathways might be playing a role to modulate GSK-3β activity in the hippocampus of rats.

Whether α7 nAChR PAMs with the differential *in vitro* profiles – type I versus type II exhibit differences in efficacy, safety, and tolerability profiles *in vivo* which needs to be elucidated. It is also another critical issue of whether and what additional advantages PAMs offer *in vivo* compared to agonists in terms of efficacy, safety, and tolerability [31]. Although further data from experimental models are needed, to the best of our knowledge, the results presented here represent the first report to elucidate the intracellular molecular mechanisms of α7 nAChR ligands is linked PDE4 activity regulating the intracellular cAMP trafficking and Akt/GSK-3β pathways in schizophrenia. These significant findings provide a necessary *in vivo* proof-of-concept that provides a platform for PAMs to

continue to be tested in a wide range of developmental animal models and encourages the further development of this novel class of drugs for the treatment of schizophrenia. All our results considered together, we suggest that α7 nAChR agonists and PAMs may be therapeutical effects on the social deficits which unmet needs of schizophrenia, and further studies should focus on the underlying molecular mechanism of beneficial effects and differences in their therapeutical profile.

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Conflicts of interest: The authors declare no conflicts of interest in this study.

Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were under the ethical standards of the institution or practice at which the studies were conducted (Erciyes University Animal Research Ethics Committee approved this study with the protocol number of "16/019").

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Highlights

1. MK-801 caused deficits in social behaviors, Akt/GSK-3β, PDE4/cAMP pathways in rats
2. α7 nAChR agonist and PAMs reversed MK-801 induced social deficits in rats
3. α7 nAChR agonist and PAMs decreased MK-801 induced GSK-3β activation
4. α7 nAChR agonist and PAMs supressed PDE4 expressions induced by MK-801
5. α7 nAChR agonist and PAMs increased MK-801 decreased cAMP levels in prefrontal cortex