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Research report

The influence of N-desmethylclozapine and clozapine on recognition memory and BDNF expression in hippocampus

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ABSTRACT

Clozapine, which is the most effective treatment option for treatment-refractory schizophrenia, has been reported to have both positive and negative effects on specific cognitive symptoms in patients with schizophrenia and in animal models of cognition. Clozapine has a major metabolite, N-desmethylclozapine (NDMC), which has been suggested to be more effective than clozapine itself to improve cognition. Enhancement of brain derived neurotrophic factor (BDNF) expression in the hippocampus has been proposed to contribute to the cognitive-enhancing effects of antipsychotic drugs. The aims of this study were to investigate the change in short and long term memory as assessed by the novel object recognition (NOR) test and BDNF expression in hippocampus produced by an acute hypoglutamatergic model of memory impairment in schizophrenia induced by administration of the NMDA receptor non-competitive antagonist, MK-801 and the ability of clozapine and NDMC to prevent the deleterious effects of MK-801. Both short (1 h) and long-term (24 h) memory were impaired in MK-801 (0.1 mg/kg) – and clozapine (5 mg/kg)-, but not NDMC (5 mg/kg)-treated rats. Neither NDMC (5 mg/kg) nor clozapine (5 mg/kg) reversed the effect of MK-801. Western blotting studies showed that BDNF levels in hippocampus were not different in rats administered MK-801 alone, clozapine or NDMC alone. These results show that in this model clozapine affects memory negatively, while NDMC does not. The absence of impairment of NOR with NDMC is consistent with previous evidence that it has a more benign effect on cognition than does the parent compound, and may support the efforts to study its effects on other cognitive functions. These findings do not provide any support for the role of BDNF in the MK-801-induced impairment in NOR or for differences between clozapine and NDMC.

1. Introduction

Clozapine is the prototypical atypical antipsychotic and has been shown to be effective in improving psychotic symptoms in most but not all schizophrenic patients who remain psychotic despite adequate trials with other antipsychotic drugs [31,43].

It was the first atypical antipsychotic drug reported to improve some domains of cognition in patients with schizophrenia [24]. It is now known that this is a shared effect with other atypicals [34,64], but it is disputed as to whether this is all due to a practice effect or is any different from that of typical antipsychotic drugs [65]. Clinical studies show a positive effect of clozapine on verbal fluency and attention, while results about its effect on working memory and secondary verbal and spatial memory have been inconclusive [46,64]. Clozapine has also been shown to have differential effect in different animal models of cognition with some studies reporting improvement [21,25,29,33] and others reporting no effect or deterioration [13,17,50,53].

Clozapine is a potent antagonist of muscarinic receptors, an effect which is known to adversely affect cognition [59,63]. On the other hand, its major metabolite N-desmethylclozapine (NDMC) has been reported to be a positive allosteric modulator of M_1 and M_4 receptors [63], and potentiate hippocampal NMDA receptor currents through M_1 receptor activation [56]. Weiner et al. [63] have suggested that the beneficial effects of clozapine on cognition may be mediated by NDMC, overcoming any negative effects of the parent compound. These observations increase the possibility that NDMC contributes to clozapine's clinical activity through modulation of both muscarinic and glutamatergic neurotransmission [56,63]. It is reported that activation of M_1 receptors by NDMC can be reduced by the presence of clozapine which is a partial agonist of M_1 and can antagonize M_1 responses induced by NDMC. Therefore, contribution of the M_1 agonist activity of

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NDMC to the useful effects of clozapine could depend on whether high levels of NDMC are present in the CNS relative to clozapine at therapeutic doses [56,63]. In accordance with the results of animal studies, high serum NDMC/clozapine ratios predicted improvement in cognitive functioning in patients with schizophrenia [63]. In a recent study clozapine/NDMC ratio was reported to be more strongly associated with cognitive impairment than clozapine and NDMC levels. NDMC agonist activity versus clozapine antagonist activity at the muscarinic receptors was suggested to explain the strength of the association of clozapine/NDMC with cognition [48]. Therefore it is reasonable to expect that direct administration of NDMC may be more effective in improving cognition than clozapine. NDMC is also a dopamine D_2 partial agonist and a D_1 agonist, while clozapine is a D_2 antagonist. Clozapine has additional pharmacology, including 5-HT_{2A} and 5-HT_{2C} inverse agonism which could influence its cognitive effects. Both drugs enhance dopamine and acetylcholine (efflux) in hippocampus and cortex [10,27,28,37].

It is well known that brain derived neurotrophic factor (BDNF) expression is related to cognitive function, especially memory, and that its levels increase with cholinergic and glutamatergic stimulation [18,67]. BDNF is highly expressed in hippocampus, a brain region known to be involved in pathophysiology of schizophrenia [52]. Post-mortem studies show a significant decrease in BDNF concentrations in cortical areas and the hippocampus of patients with schizophrenia [15]. BDNF has also been reported to be decreased in the serum of schizophrenic patients [60]. In addition, a genetic link between BDNF and schizophrenia is suggested by the evidence that a polymorphism of BDNF gene is associated with schizophrenia [57]. Results from the animal models also supported the hypothesis that BDNF plays a role in the pathophysiology of schizophrenia [2,19,40].

The results regarding the effects of typical and atypical antipsychotics on BDNF expression are contradictory. There are reports of increase in BDNF expression in hippocampus after treatment with atypical antipsychotics, although these results could not be repeated by others [1,3,9]. Both olanzapine and quetiapine was found to prevent the decrease in BDNF caused by the NMDA receptor non-competitive antagonist, MK-801 [19,20]. Regarding the effects of clozapine on BDNF, there are some clinical studies which report a higher levels of serum BDNF in chronic schizophrenic patients on clozapine compared to the ones on typical antipsychotics and risperidone [22,58,66], whereas the results of animal studies are inconsistent. Some report an increase in the expression of BDNF mRNA in CA1, CA3 and dentate gyrus of hippocampus [3], while others report no change [4,38,62], or even a decrease [40].

To our knowledge, there is no study exploring the effect of NDMC on BDNF expression in rats. We postulated that NDMC may have positive effects on cognition by increasing expression of BDNF in hippocampus. To test cognition, we employed novel object recognition test (NOR), since it has become a widely used tool to screen the procognitive effects of new antipsychotics in animal models [68]. Moreover, pharmacological models of schizophrenia have been reported to disrupt NOR performance [11,68]. It is a non-spatial nonaversive test of recognition memory based on rodents' tendency to spend more time exploring the novel object over the familiar object, which is interpreted as reflecting the rodent's memory for the familiar object and it's desire to explore a novel object [12].

The goals of this study were to quantify the change in NOR test performance and BDNF expression in rat hippocampus, and to compare the effects of NDMC and clozapine in rats given MK-801. We hypothesized that MK-801 administration would cause a decrease in BDNF expression and deterioration in memory, while both clozapine and NDMC administration would prevent this, with NDMC being more effective on both measures than clozapine.

2. Materials and methods

2.1. Animals

Male Wistar rats weighing 200–350 g were used as experimental subjects. All animals were maintained in groups of 4 at 12 h light/dark cycles in a temperature and humidity-controlled room, with food and water available *ad libitum*. The experimental procedures were reviewed and approved by Hacettepe University Ethics Commitee for Experimental Animal Research (2007-5).

2.2. Drugs

MK-801 hydrogen maleate was purchased from Sigma (USA), and dissolved in saline. NDMC was a gift from ACADIA (San Diego, USA) and clozapine was a gift from Adeka (Samsun, Turkey). Both drugs were dissolved in acidified water, prepared from .1 mol/l hydrochloric acid, diluted with water and titrated with .1 mol/l sodium hydroxide to pH 5.5 [26].

2.3. Behavioral procedures

The NOR test was performed according to the method described previously [6,11,16]. The experiments were done in a black open field box with dimensions of 40 cm × 40 cm × 40 cm. Rats were habituated to the box for 10 min per day for two days with no objects present. Twenty-four hours after habituation, training was done by placing individual rats for 10 min into the box, in which two identical objects (F1, F2, Lego toys) were put symmetrically in two adjacent corners, 10 cm from the walls. The short-term memory test was given 1 h after the training. The rats explored the open field for 5 min in the presence of one familiar (F) and one novel (N) object. The objects had similar textures and sizes, but different shapes. Between trials the objects and the box were washed with 70% ethanol solution. The long-term memory was tested 24 h after the training session. The same rats were allowed to explore the field for 5 min in the presence of the familiar object and another novel object. An animal was considered to be exploring the object when sniffs or touchs the object with the nose and/or forepaws. The animals were videotaped in all sessions. The discrimination index calculated for each animal was expressed by the ratio $T_N/(T_F + T_N)$ [T_F = time spent exploring the familiar object F; T_N = time spent exploring the novel object N].

2.4. Experimental procedure-1

The first experiment included 6 groups of rats with 5 rats in each group. All injections were done intraperitonealy with 1 ml/kg injection volume. The drugs were administered at doses which would not affect the NOR test due to side effects such as sedation, ataxia etc. [11,25,35]. The rats in the first two groups received either saline or MK-801 (0.1 mg/kg) 20 min before NOR training trial. Rats in the other four groups received clozapine (5 mg/kg) plus saline, clozapine (5 mg/kg) plus MK-801 (0.1 mg/kg), NDMC (5 mg/kg) plus saline, or NDMC (5 mg/kg) plus MK-801 (0.1 mg/kg). Clozapine (5 mg/kg) or NDMC (5 mg/kg) were injected 30 min before saline or MK-801 (0.1 mg/kg). Twenty min after the injection of MK-801 or saline, NOR training trial was performed. NOR test was performed with inter-trial intervals of 1 h (Test-1) and 24 h (Test-2) to assess short and long term memory. After completion of NOR Test-2, all rats were decapitated and the hippocampi were extracted for western blotting to measure BDNF expression.

2.5. Experimenal procedure-2

Considering the possibility that higher doses of drugs may influence BDNF expression in a different way [8,19,20], the BDNF expression was also studied in a second experiment in which higher doses of the drugs were administered and the NOR test was not included. Four groups were studied: saline ($n = 5$), MK-801 (1 mg/kg) ($n = 3$), clozapine (30 mg/kg) + MK-801 (1 mg/kg) ($n = 4$), and NDMC (30 mg/kg) + MK-801 (1 mg/kg) ($n = 4$). Clozapine or NDMC were administered 30 min before saline or MK-801. All rats were decapitated 24 h after injection. BDNF expression in the hippocampi was assessed with western blotting.

2.6. Western blotting

The rats were decapitated after being anesthesized by intraperitoneal injection of chloral hydrate. Brains were quickly removed, frozen in dry ice, and kept at −80 °C until use for western blotting. The hippocampi were dissected. Samples were homogenized in radioimmunoprecipitation (RIPA) buffer which included 2% protease inhibitor cocktail. The homogenates were centrifuged at +4 °C and 14,000 rpm for 15 min. Aliquots from hippocampal homogenates were separated by SDS-PAGE on 10% Novex Bis-Tris (Invitrogen) gel and transferred electrophoretically to a polyvinylidene flouride (PVDF) membrane. After occupation of non-specific binding sites with blocking solution (Tris Buffered Saline, 0.1% Tween-20, 5% casein), the membranes were incubated overnight at +4 °C with primary anti-BDNF antibody (rabbit, polyclonal, 1/500; [N-20] sc-546, Santa Cruz Biotechnology, USA). The membranes were washed with Tris-buffered saline containing 0.1% Tween 20 and then incubated with the secondary antibody (alkaline phosphatase-conjugated goat